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(54) Title: TUMOR TARGETING WITH L-ENANTIOMERIC OLIGONUCLEOTIDE CONJUGATES OF IMMUNOREAGENTS AND OF CHELATED RADIONUCLIDES

(57) Abstract

The present invention is directed to a non-radioactive targeting immunoreagent that comprises an immunoreactive group, one or more non-self-associating L-enantiomeric oligonucleotide sequences, and one or more linking groups, and to a radioactive targeting immunoreagent that comprises an L-enantiomeric oligonucleotide sequence that is complementary in sequence to and capable of hybridization with one or more fragments of a non-self-associating L-enantiomeric oligonucleotide sequence, one or more chelating agents, one or more linking groups, and one or more radionuclides. The present invention is also directed to pharmaceutical compositions comprising one or more of the above-described immunoreagents and a pharmaceutically acceptable carrier. The present invention is further directed to methods for treating and imaging disease sites in patients.

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TUMOR TARGETING WITH L-ENANTIOMERIC OLIGONUCLEOTIDE CONJUGATES OF IMMUNOREAGENTS AND OF CHELATED RADIONUCLIDES

Field of the Invention

This invention relates to sequential targeting and delivery of immunoreagent compositions which find particular utility in the therapy and diagnostic imaging of cancer by means of a tumor targeted sequential delivery system comprising a primary non-radioactive targeting immunoreagent and a secondary radioactive delivery agent. This invention also relates to novel methods for the attachment of L-enantiomeric oligonucleotides, complementary L-enantiomeric oligonucleotides, and chelates, to proteins, and to bifurcated tumor targeting and delivery vectors for the treatment and diagnostic imaging of tumors.

Background of the Invention

The various, currently available, radiolabeled immunoreactive proteins and methods which are employed in diagnostic imaging and targeted therapeutic applications suffer from certain disadvantages. For example, radioimmunotherapy and diagnostic imaging with the various currently available radiopharmaceuticals which include radionuclide-containing immunoreactive proteins can be less than optimal because these radiopharmaceuticals may bind to non-target normal tissue. This binding can result in undesirable toxicity to normal tissue during therapeutic applications as well as in high background signals during diagnostic imaging applications. Inefficient covalent bonding of the radioactive component with protein in conjugate preparation can be another problem due to release of the radioactive component which may then deposit in healthy tissue. Also, long plasma half-lives of currently available radionuclide-containing immunoreactive

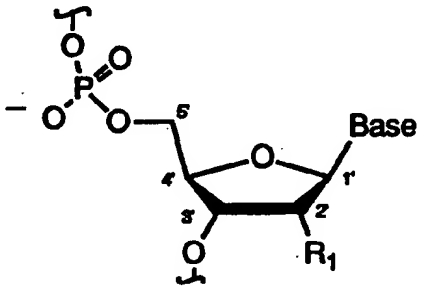
proteins and slow clearance of radionuclide from the body can result in prolonged exposure of normal tissue to damaging effects of radiation and can produce unacceptable toxic effects in otherwise normal and disease free tissues in the body, especially in those tissues and cells most sensitive to radiation damage, e.g., the stem cells of the bone marrow and gastrointestinal tract. While the number of ionic radionuclides that can be associated with an immunoreactive protein is restricted by the number of sites of chelation available, an increase in that number which can be achieved by increasing the number of chelating agents conjugated to the protein can produce a decrease in the immunoreactivity of the protein. This can limit the number of such chelating agents that can be attached to the protein. The number of chelating agents that can be attached to an immunoreactive protein is also limited by the number of available groups such as, for example, amino groups suitable for use in attachment of the chelating agents and by the potential immunogenicity of the thus modified protein which, being highly derivatized, could be recognized by a host immune system as being haptenated.

It is an object of the present invention to overcome some of the aforementioned disadvantages of the currently available radiolabeled immunoreactive proteins.

Nucleic acids in the form of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) encode and transfer genetic information for the cellular synthesis of proteins and enzymes. Naturally occuring nucleic acids are composed of nucleosides such as 2'-deoxyadenosine (dA), 2'-deoxyguanosine (dG), 2'-deoxycytidine (dC), and thymidine (T) in DNA and of adenosine (A), guanosine (G), cytidine (C), and uridine (U) in RNA. Naturally occuring modified nucleosides such as those containing

2'-O-methylribosyl groups and those containing bases such as N^4,N^4-dimethyladenine and N^7-methylguanidine are found in messenger, transfer, and ribosomal RNA. Internucleosidyl phosphodiester bonds link nucleosides at the oxygen of the 3'-hydroxyl group of a D-ribose or of a D-deoxyribose sugar moiety in one nucleoside to the oxygen of a 5'-hydroxyl group in a D-ribose or D-deoxyribose sugar moiety in another nucleoside in RNA and in DNA, respectively. Separate chains of nucleic acids interact with one another via hydrogen bonds formed between complementary pairs of nucleosidyl purine and pyrimidine bases: adenine with thymine and guanine with cytosine in DNA, and adenine with uracil and guanine with cytosine in RNA. When the sequences of bases in two separate oligonucleotide strands or in two regions of a single strand are complementary to each other, the complementary sequences can hybridize with each other via hydrogen bonds between complementary base pairs to form a right-handed double helix with a B-type conformation, the two phosphate-ribose ester backbones of which are antiparallel.

Structure I



D-Enantiomers
D-DNA: R_1 = H
D-RNA: R_1 = OH

In naturally occurring DNA each nucleoside unit is a D-enantiomer whose structure is defined by the chirality of the D-deoxyribose ring which has substituents at the 1'-(b), 3'-(a), and 4'-(b) positions as represented schematically in Structure I wherein R_1 is H and "Base" represents an adenine, guanine, thymine or cytosine moiety. In naturally occurring RNA each nucleoside unit is also a D-enantiomer whose structure is defined by the chirality of the D-ribose ring which has substituents at the 1'-(b), 2'-(a), 3'-(a), and 4'-(b) positions as represented schematically in Structure I wherein R_1 is OH and "Base" represents an adenine, guanine, uracil or cytosine moiety. In both naturally occurring DNA and RNA, the phosphate diesters and the bases do not comprise chiral centers.

As noted by P. S. Miller in Bioconjugate Chem. 1990, 1, 187-191, synthetically prepared oligonucleotides composed of naturally configured D-enantiomers have been used as primers for nucleic acid polymerizing enzymes, as synthons in the construction of artificial genes for the preparation of proteins by recombinant DNA techniques, and as diagnostic probes to detect and characterize cellular nucleic acid sequences. In addition, synthetically prepared oligonucleotides have been investigated for use in the control of gene expression in living cells, and as therapeutic agents in the inhibition of viral replication and in the treatment of cancer. These applications rely on the sequence specific complementary binding properties of the synthetic oligonucleotides with natural D-enantiomeric oligonucleotide target sequences.

Synthetically prepared oligonucleotides composed of naturally configured D-enantiomers can be generated by a variety of methods, the currently most useful of which include solid phase synthesis via phosphoramidite intermediates and solid phase synthesis via H-

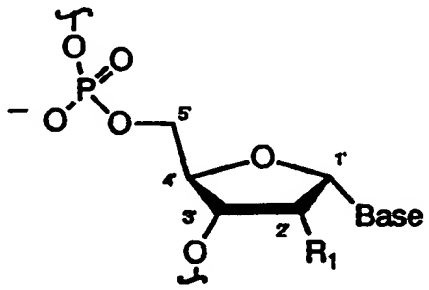
phosphonate intermediates as described by E. Uhlmann and A. Peyman in Chemical Reviews, 1990, 90, 544. In the phosphoramidite method, a 5'-hydroxyl group of a growing DNA oligomer chain of D-enantiomers having amide protecting groups on the exocylic amine groups of the bases therein and which is attached to a solid phase support reacts with an activated D-nucleoside 5'-O-dimethoxytrityl-3'-(2-cyanoethyl N,N-diisopropyl)-phosphoramidite in the presence of 1H-tetrazole as a catalyst. Commonly used protecting groups include the benzoyl group for the protection of the exocyclic amino groups of adenine and cytosine and the isobutyryl group for the protection of the exocyclic amino group of guanine. Any unreacted 5'-hydroxyl groups are capped with acetate groups by reaction with acetic anhydride in the presence of 4-N,N-dimethylaminopyridine. The resulting phosphite is then oxidized with iodine to form a phosphotriester. The 5'-O-dimethoxytrityl group is removed under acid conditions using dichloroacetic acid, and the reaction sequence is repeated using another activated D-nucleoside 5'-O-dimethoxytrityl-3'-(2-cyanoethyl N,N-diisopropyl) phosphoramidite. At the end of the synthetic sequence, the oligonucleotide is freed from amide protecting groups and cleaved from the support by treatment with ammonium hydroxide. The oligonucleotide is then purified or isolated by methods such as precipitation, electrophoresis, or chromatography.

Many in vivo applications of synthetic D-enantiomeric oligonucleotides are limited by the rapid destruction of the oligomers by nucleases which are encountered both extracellularly and intracellularly. As reviewed by J. Goodchild in Bioconjugate Chem. 1990, 1, 165, strategies to introduce resistance to nuclease activity included modification of the nucleoside bases, and of the phosphate and ribose components of the

polymer backbone. All of these were done with the need to maintain the ability of the modified oligonucleotide to hybridize with a naturally occuring D-enantiomeric DNA or RNA target sequence or to bind with an enzyme that uniquely recognizes the D-enantiomer.

The effects on the properties of D-oligonucleotides of a single modification of oligonucleoside chirality such as can be achieved through inversion at the 1'-ribose site to form an a-anomer, represented in Structure II, have been studied by Bloch et al in Gene, 1988, 72, 349. These workers have found that alpha-anomeric DNA hybridizes with complementary beta-anomeric RNA, the naturally occurring enantiomeric oligomer, to form an a:b DNA:RNA hybrid, and that the hybrid is nuclease resistant.

Structure II



α-Anomers

α-DNA: $R_1 = H$

α-RNA: $R_1 = OH$

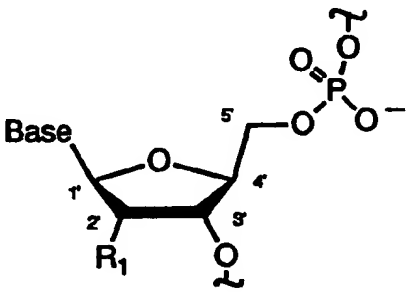
Thuong and Chassignol in Tetrahedron Letters, 1988, 29, 5905 reported the solid phase synthesis employing 2-cyanoethyl phosphoramidite synthons of oligo-a-deoxynucleotides containing a-d-T, a-d-C, and a-d-A covalently linked at the 5'-phosphate to 2-methoxy-6-chloro-9-(5-hydroxypentylamino)acridine. Analogous oligo-a-deoxynucleotides without the acridine moiety

were reported to be resistant to nucleases and to form hybrids with natural D-RNA that were more stable than those with b-deoxynucleotides. Morvan et al in Nucleic Acid Research, 1988, 16, 833, reported the preparation of oligo-a-deoxynucleotides containing a-d-T, a-d-C, a-d-G, and a-d-A which formed double helix hybrids or duplexes with complementary a and b strands with parallel and antiparallel polarity, respectively. These duplexes retained specific Watson-Crick base pairing specificity, and the heteroduplexes were shown to belong to the B-DNA family. They also showed higher thermal stability when compared to the corresponding naturally occurring bb-DNA duplexes. Again, however, the target oligomer was a naturally occurring D-oligomer.

Complete inversion of all chiral sites of the D-enantiomer in Structure I provides the mirror image, non-naturally occurring L-enantiomer which is represented in Structure III. In the absence of a chiral environment or a chiral reagent such as an enzyme which can distinguish between D- and L-enantiomers, the L-enantiomer is identical in chemical reactivity to the D-enantiomer. Urata et al in J. Am. Chem. Soc. 1991, 113, 8174 reported the synthesis (via the 2-cyanoethyl phosphoramidite method) and properties of the self-complementary L-DNA oligomer duplex, L-d(CGCGCG) and compared them to those of the mirror image, natural D-d(CGCGCG) oligomer. The unnatural all-L-duplex was resistant to digestion by nuclease P-1 while the natural all-D-duplex was rapidly degraded to the component D-nucleotides and 5'-end deoxycytidine. As expected, both the DD- and the LL-duplexes exhibited identical HPLC retention times and their circular dichroism (CD) spectra were mirror images of one another. In 0.1 M NaCl, the D-enantiomer showed the CD profile of a standard B form while the L-enantiomer exhibited the same magnitude but opposite sign at all wavelengths.

This implied the existance of a mirror image B form helix with left-handed double-helical conformation. In 4 M NaCl, the D-enantiomer showed the profile of a left-handed Z form while the L-enantiomer exhibited the same magnitude but opposite sign at all wavelengths indicating the existance of a mirror image Z form with a right-handed double helical conformation. The CD spectra of both the all D-duplex and all L-duplex forms exhibited the same B to Z conformational transition (with opposite signs) as a function of salt concentration as well as the same temperature dependence under both low and high salt concentrations. It was concluded that the D- and L-DNA have the same type and strength of hydrogen bonding and base stacking interactions, and that the structure of the L-DNA is the exact mirror image of that of the D-DNA. This all L-enantiomeric oligomer, being self-complementary, could not be used to target either a separate natural D- or unnatural L-enantiomer.

Structure III



L-Enantiomers
L-DNA: R_1 = H
L-RNA: R_1 = OH

Fujimori et al in Nucleic Acid Research, 1990, 22, 97 reported the synthesis of 9-(2-deoxy-b-L-*erythro*-

pentafuranosyl)-9H-purin-6-amine, L-dA, and the hexameric L-DNA, L-d(AAAAAA). They observed L-d(AAAAAA) to have resistance to bovine spleen phosphodiesterase while D-d(AAAAAA) was completely degraded. They also noted that the L-d(AAAAAA) formed a complex with a natural D-RNA enantiomer, D-poly(U), which was weaker relative to that formed between the enantiomeric natural D-d(AAAAAA) and D-poly(U). No complex was formed between the unnatural L-d(AAAAAA) and the natural DNA enantiomer D-poly(T). These results suggested that while L-enantiomeric DNA has the ability to distinguish complementary RNA from DNA, the unnatural L-DNA:D-RNA complexes are not nearly as strong as the natural corresponding D-DNA:D-RNA complexes.

Although L-DNA has been shown to hybridize *in vitro* with natural enantiomeric D-RNA and to be stable to nuclease resistance, in practice such a duplex formation with targeted D-RNA is not readily achievable *in vivo* since it is necessary for the L-DNA to cross at least one cell membrane to find the intact D-RNA. Furthermore, natural D-RNA has a relatively short lifetime in the presence of naturally occurring nuclease enzymes.

Summary of the Invention

The present invention is directed to a non-radioactive targeting immunoreagent that comprises a tumor antigen recognizing moiety, one or more L-enantiomeric oligonucleotides comprising non-self-associating L-enantiomeric oligonucleotide sequences, and one or more linking groups.

The present invention is also directed to a radioactive targeting immunoreagent that comprises an L-enantiomeric oligonucleotide comprising an L-enantiomeric oligonucleotide sequence that is complementary in sequence to and capable of

hybridization with one or more fragments of a non-self-associating L-enantiomeric oligonucleotide sequence, one or more chelating agents, one or more linking groups and one or more radionuclides.

The present invention is also directed to pharmaceutical compositions comprising one or more of the above-described immunoreagents and a pharmaceutically acceptable carrier.

The present invention is further directed to methods for treating and imaging disease sites such as tumor sites in a patient. Said methods comprise administration to the patient of an effective amount of the above-described non-radioactive targeting immunoreagent followed at an effective time interval by an effective amount of the above-described radioactive targeting immunoreagent.

The present invention provides many advantages compared to conventional targeting immunoreagents. For example, the non-radioactive targeting immunoreagent can accumulate at a tumor site in vivo while it is not accumulated at normal tissue sites.

The in vivo residence half life of the non-radioactive targeting immunoreagent is long enough to permit its accumulation at a tumor site.

The in vivo residence half life of the radioactive targeting reagent is shorter than the residence half life of the non-radioactive targeting immunoreagent.

The portion of the radioactive targeting reagent that does not hybridize to tumor associated non-radioactive targeting reagent is rapidly cleared from the patient.

With respect to the same degree of modification of a targeting immunoreagent by a directly labeled radionuclide or a chelate containing a radionuclide, an amplification of the number of radionuclides per site of

modification per targeting immunoreagent can be obtained.

A segment of the complementary sequenced L-enantiomeric oligonucleotide of the non-radioactive targeting immunoreagent and a segment of the L-enantiomeric oligonucleotide of the radioactive targeting reagent can hybridize in vitro and in vivo.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent will not hybridize with isomeric, complementary sequenced, naturally occurring D-enantiomeric oligonucleotides.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent are stable to nuclease activity.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent and the hybrid formed between the complementary L-enantiomeric oligonucleotides will not bind to enzymes which are specific for binding to isomeric D-enantiomeric oligonucleotides.

The hybrid formed between complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent is stable to nuclease activity.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent do not self hybridize.

The non-radioactive targeting immunoreagent and the radioactive targeting reagent can comprise a wide variety of spacing, linking, and chelating groups, L-enantiomeric oligonucleotide sequences and radionuclides.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent can comprise L-enantiomeric oligonucleotide sequences which can be tandemly linked by spacing groups, wherein a segment of each L-enantiomeric oligonucleotide sequence can hybridize with a segment of the radioactive targeting reagent.

The complementary sequenced L-enantiomeric oligonucleotides of the non-radioactive targeting immunoreagent can be linked to an antibody by means of either a 5'- or a 3'-substituent such as a 5'-amine or 3'-amine.

Reagents are provided that have a specificity for tumors and a wide variety of compositions can be prepared in accordance with the present invention.

A particular advantage of the present invention is that L-enantiomeric oligonucleotide sequence lengths and spacing groups can be selected such that on hybridization of two complementary radioactive targeting reagent L-enantiomeric oligonucleotide sequences to a single L-enantiomeric oligonucleotide-containing strand comprising adjacent, tandemly linked L-enantiomeric oligonucleotide sequences of a non-radioactive targeting immunoreagent, the proximal end groups of the sequences of two such radioactive targeting moieties are orthogonal to each other because of their relative spacial configuration on the double stranded helix so formed.

Other advantageous features of this invention will become readily apparent upon reference to the following description of the preferred embodiments.

Description of the Preferred Embodiments

This invention describes various novel bioconjugates which possess utility in therapeutic and diagnostic imaging compositions and methods. This

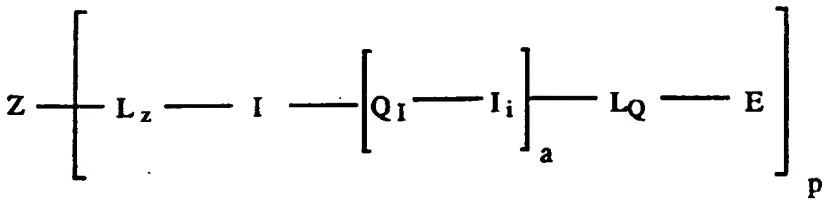
invention further describes novel methods of preparing bioconjugates by the attachment of various L-enantiomeric oligonucleotide sequences to chelating agents, preferably terpyridine containing chelating agents, and to immunoreagents such as proteins, antibodies, and receptors.

In particular, this invention describes novel bioconjugates useful for sequential targeting and amplified delivery of novel radioactive immunoreagent compositions which find particular utility in therapeutic and diagnostic imaging compositions and methods.

More particularly, this invention describes the preparation and use of targeting immunoreagents that comprise a tumor antigen recognizing moiety, one or more L-enantiomeric oligonucleotides comprising non-self-associating L-enantiomeric oligonucleotide sequences, and one or more linking groups. These targeting immunoreagents react with a radioactive sequential targeting reagent that comprises an L-enantiomeric oligonucleotide sequence that is complementary in sequence to and capable of hybridization with one or more fragments of the said non-self-associating L-enantiomeric oligonucleotide sequences, one or more chelating agents, one or more linking groups, and having one or more radionuclides associated therewith. Most preferably, the L-enantiomeric nucleotides are L-enantiomers of natural D-deoxyribonucleotides.

In a preferred embodiment, the above-described targeting immunoreagents form a compound that comprises moieties represented by the structure IV:

Structure IV



wherein:

Z is the residue of an immunoreactive protein;

L_Z and L_Q are independently a chemical bond or a linking group;

I is an L-enantiomeric oligonucleotide comprising a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family comprising from 12 to about 30 L-enantiomeric nucleotide units, and provided that contiguous sequences of six or more L-enantiomeric nucleotide units of said L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure IV;

Q_I is a spacing group;

a is 0 or an integer from 1 to about 6;

I_i is an L-enantiomeric oligonucleotide comprising a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units, a contiguous sequence therein comprising a portion of I;

E is an end capping group; and

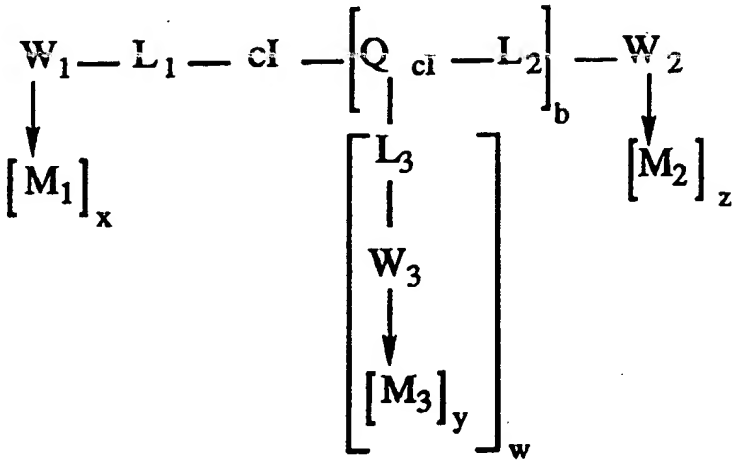
p is an integer from 1 to about 10.

More preferably, a is an integer from 1 to about 6.

In another preferred embodiment, the above-described targeting reagent comprises moieties represented by the structure V:

described targeting reagent comprises moieties represented by the structure V:

Structure V



wherein:

cI is a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family comprising from 12 to about 30 L-enantiomeric nucleotide units, where the nucleotide sequences of said homologs are complementary to the nucleotide sequences of members of the set of L-enantiomeric oligonucleotides in a co-administerable targeting immunoreagent, and where contiguous sequences of six or more L-enantiomeric nucleotide units of said complementary L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure V;

Q_{cI} is a spacing group;

L_1, L_2, and L_3 are independently a chemical bond or a linking group;

W_1, W_2, and W_3 are each a residue of a chelating group;

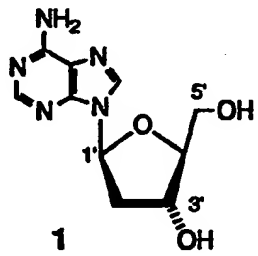
M_1, M_2, and M_3 comprise elements with oxidation states equal to or greater than +1, and at least one of M_1, M_2 and M_3 is a radionuclide;

x, y, and z are independently zero or one provided that at least one of x, y, or z is one; and

w and b are independently zero or an integer from 1 to about 4.

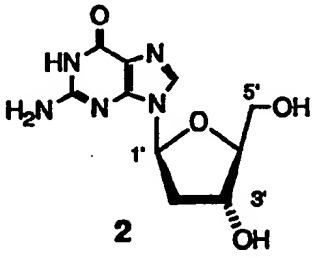
As used herein, an L-enantiomeric nucleotide unit is defined as the mirror image of the naturally occurring, isomeric D-enantiomer nucleotide unit; an L-enantiomeric nucleoside is defined as the mirror image of the naturally occurring, isomeric D-enantiomeric nucleoside; and an L-enantiomeric oligonucleotide sequence is defined as the mirror image of the naturally occurring, isomeric D-enantiomeric oligonucleotide sequence.

Non-limiting examples of L-enantiomeric nucleosides are set forth below.



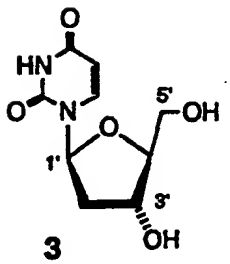
1

2-deoxy-L-adenosine, L-dA



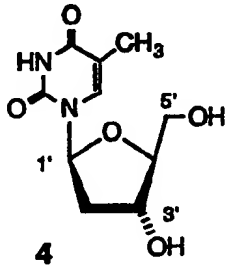
2

2-deoxy-L-guanosine, L-dG



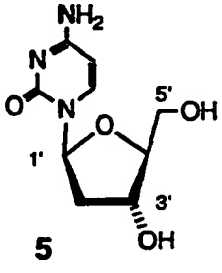
3

2-deoxy-L-uridine, L-dU



4

2-deoxy-L-thymidine, L-dT



5

2-deoxy-L-cytidine, L-dC

Methods of synthesis of L-enantiomeric oligonucleotides from L-enantiomeric nucleotide intermediates are identical to those used in the synthesis of mirror image D-enantiomeric oligonucleotides from isomeric D-enantiomeric nucleotide intermediates. Examples of such methods include those discussed in "Chemistry of Nucleosides and Nucleotides", edited by Leroy B. Townsend, Plenum Press, N.Y., 1988. The synthesis of L-enantiomeric oligonucleotides by such methods requires the use of L-enantiomeric reagents such as L-enantiomeric nucleoside and nucleotide derivatives. The preparations of various L-enantiomeric nucleosides are well documented by Robins, M. et al (1970), J. Org. Chem., 35, p636-639; Holy, A. (1972), Coll. Czechoslov. Chem. Commun., 37, p4072-4087; Visser, G. et al (1986), Rec. Travaux Chim. Pay-Bas, 105/12 p528-537; Anderson, D. et al (1984), Nucleosides and Nucleotides, 3/5, p499-512; Uhlmann, E. et al (1990), Chem. Review, (1990), 90/4 p543-584; Smejkal, I. et al (1964) Coll. Czecholsov.Chem. Commun., 29, p2809-2813, and others.

Of the methods known in the art for the synthesis of oligonucleotides, a preferred method of synthesis of L-enantiomeric oligonucleotides of this invention comprises solid phase synthesis utilizing L-enantiomeric nucleotide phosphoramidite intermediates which contain blocking groups on the hydroxyl groups therein. The blocking of a 5'-hydroxyl group in a ribonucleoside moiety and in a deoxyribonucleoside moiety is preferrably done with an acid labile trityl group such as, for example, a monomethoxytrityl group (sometimes hereafter referred to as an MMT group) or a dimethoxytrityl group (sometimes hereafter referred to as a DMT group) employing the respective trityl chlorides as reagents. The deblocking of such a trityl-blocked 5'-hydroxyl group is preferably done with an

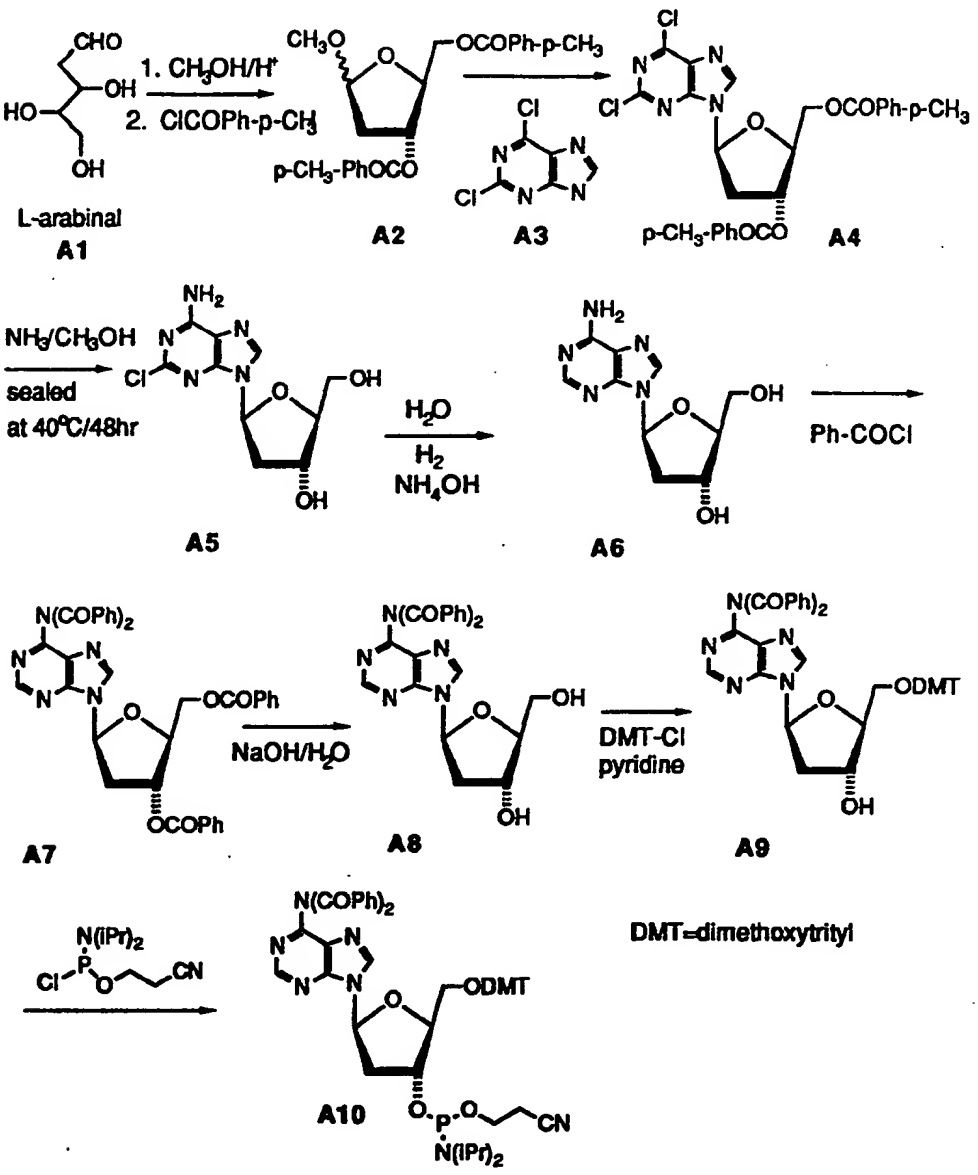
acid such as, for example, acetic acid in water, a chloroacetic acid in a solvent such as dichloromethane, or benzenesulfonic in a solvent such as chloroform or methanol as a reagent. The blocking of a 2'-hydroxyl group in a ribonucleic acid moiety is preferably done with a silyl chloride reagent such as, for example, t-butyldimethylchlorosilane which reacts with a ribosyl 2'-hydroxyl group to form a t-butyldimethylsilyl ether. The deblocking of such a t-butyldimethylsilyl ether can be achieved by treatment with, for example, sodium hydroxide dissolved in a solvent such as, for example, methanol.

Preferred L-enantiomeric nucleotides are L-2-deoxyribonucleotides, and preferred L-enantiomeric oligonucleotides are oligo-L-2-deoxyribonucleotides. Derivatives of L-enantiomeric deoxyribonucleotides that are useful in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotides of this invention are L-enantiomeric deoxyribonucleosides such as, for example, derivatives of compounds 1 to 5 which have the 5'-OH in each blocked, for example, with a dimethoxytrityl (DMT) group, each of which is activated at the 3'-OH for phosphate bond formation in the oligonucleotide, for example, by treatment with 2-cyanoethyl-N,N-diisopropylphosphoryl chloride to produce the respective 3'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite intermediates. These are utilized for the synthesis of oligo-L-deoxyribonucleotide moieties, for example, on a solid phase, automated DNA synthesizer.

A preferred method of preparing a blocked 2'-deoxy-L-adenosine 3'-O-phosphoramidite derivative, A10, that is suitable for use in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotide materials of this invention is outlined in Scheme 1. 1-O-Methyl-3,5-di-O-p-toluyl-2-deoxy-L-erythro-pentofuranose (A2 in Scheme 1) is prepared from L-arabinal (A1) by treatment

with p-toluoyl chloride and methanol according to the method described by Smejkal, I. et al (1964), Coll. Czechoslov. Chem. Commun., 29, p2809-2813. Fusion of A2 and 2,6-dichloropurine (A3) provides 2,6-dichloro-9-(3,5-di-O-p-toluyl-2-deoxy-alpha-L-erythro-pentofuranosyl)purine (A4). Treatment of A4 with methanol and ammonia selectively aminates the purine at position 6 and concurrently deblocks the acylated hydroxyl groups at the 3'- and 5'-positions. The remaining chloride is removed by hydrogenolysis to afford the desired L-nucleoside, L-dA, (A6). The exocyclic amine of A6 is then blocked by diacylation with benzoyl chloride to form A7 which contains two benzoate esters in addition to the blocked amine. The benzoate esters are removed by saponification with sodium hydroxide in water to form A8. This diol is then treated with dimethoxytrityl chloride (available from Aldrich Chemical Company) to form A9 which has a DMT ether at the 5'-position. A9 is then treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (available from Aldrich Chemical Company) to provide A10, the desired diprotected 5'-O-dimethoxytrityl-2'-deoxy-L-adenosine 3'-O-2-cyanoethylphosphoramidite. A10 is used as an intermediate in the synthesis of L-oligonucleotide sequences of the compositions of Structure IV and Structure V of this invention. Production of such sequences can be done, for example, using an automated oligonucleotide synthesizer using procedures described by the manufacturer for the synthesis and purification of isomeric D-oligonucleotides.

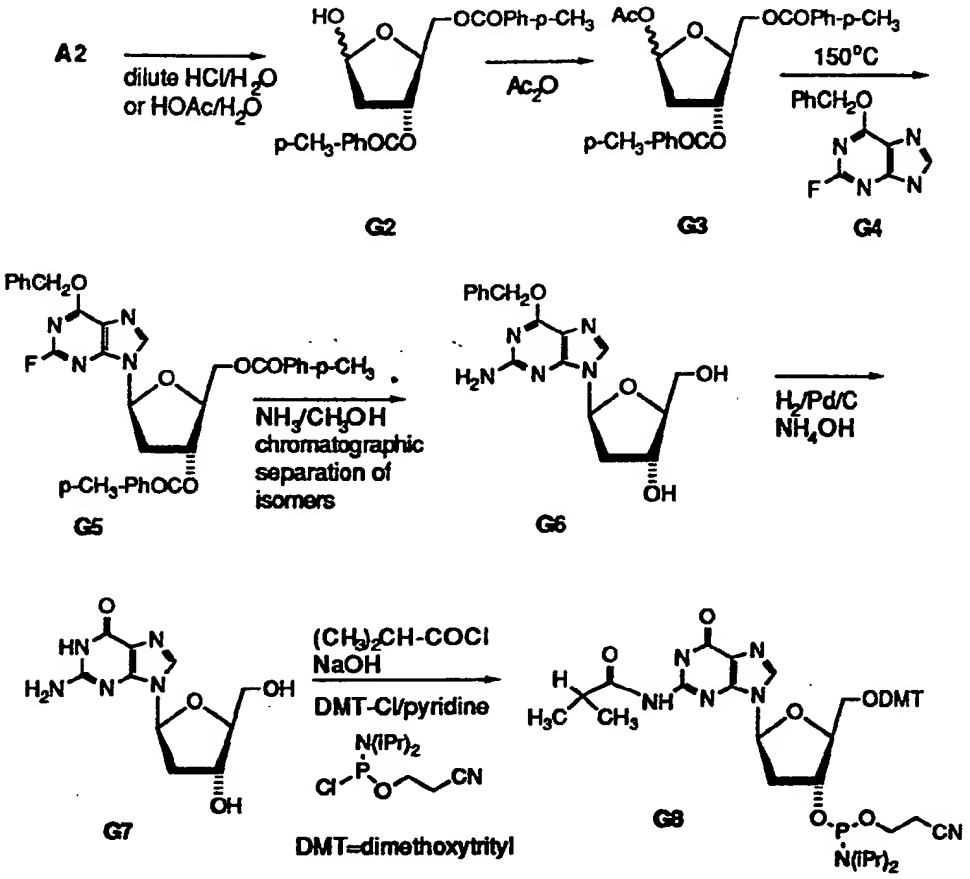
Scheme 1 Preparation of a Blocked L-deoxyadenosine phosphoramidite



A preferred method of preparing a blocked 2'-deoxy-L-guanosine 3'-O-phosphoramidite derivative, G8, that is suitable for use in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotide materials of this invention is outlined in Scheme 2. 1-O-Methyl-3,5-di-O-p-toluyl-2-deoxy-L-erythro-pentofuranose (A2 in Scheme 1) is prepared from L-arabinal (A1) by treatment with p-

toluoyl chloride and methanol according to the method described by Smejkal, I. et al (1964), Coll. Czechoslov. Chem. Commun., 29, p2809-2813. The methyl acetal (A2) is hydrolyzed in dilute hydrochloric acid to provide the hemiacetal (G2) which is acylated with acetic anhydride to form 1-acetyl 3,5-di-O-p-toluyl-beta-L-erythro-pentofuranose (G3). Fusion of G3 and 2-fluoro-6-benzyloxypurine (G4) provides G5 which is then treated with alcoholic ammonia to provide the desired product (G6) which is purified by chromatography. The 6-benzyl protecting group of G6 is removed by hydrogenolysis with palladium on carbon in ammonium hydroxide to yield 2-amino-9-(2-deoxy-L-erythro-pentofuranosyl)purin-6-one (G7; L-dG). The exocyclic 2-amino group of G7 is then protected by acylation with isobutyryl chloride, the 5'-hydroxyl group is protected as a DMT ether using dimethoxytrityl chloride, and the 3'-hydroxyl is treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite to provide G8, the desired diprotected 5'-O-dimethoxytrityl-2'-deoxy-L-guanosine 3'-O-2-cyanoethylphosphoramidite. G8 is used as an intermediate in the synthesis of L-oligonucleotide sequences of the compositions of Structure IV and Structure V of this invention. Production of such sequences can be done, for example, using an automated oligonucleotide synthesizer using procedures described by the manufacturer for the synthesis and purification of isomeric D-oligonucleotides.

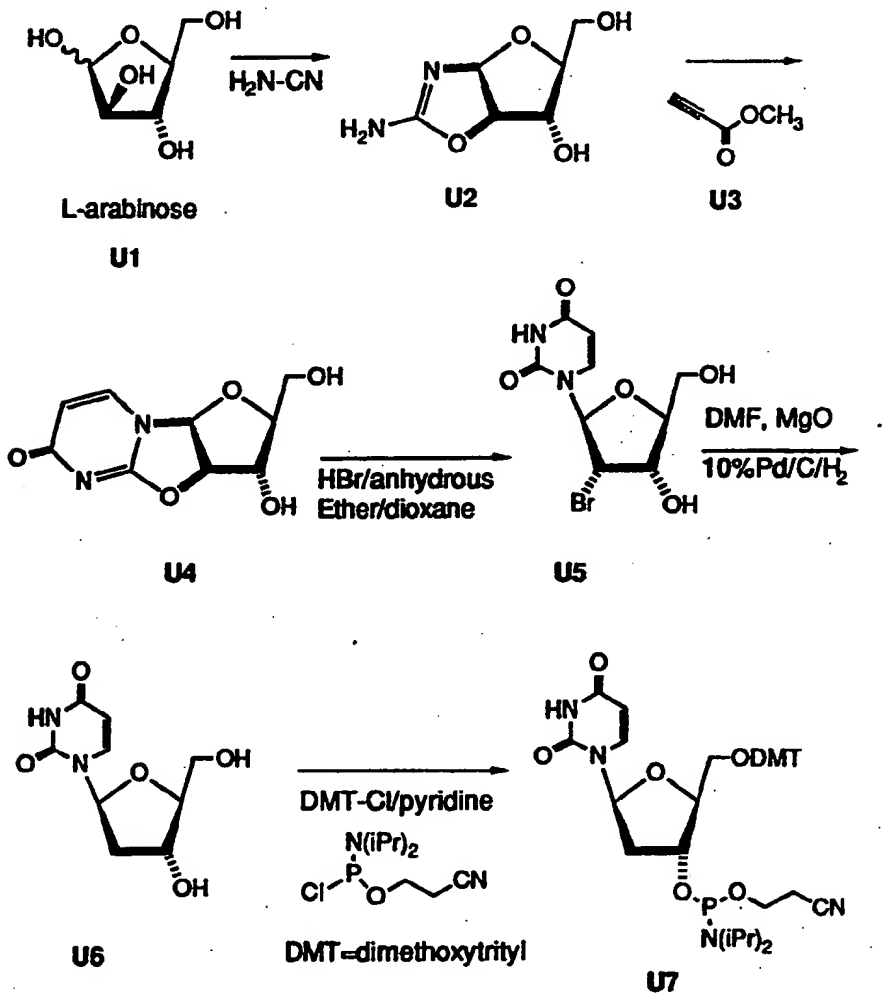
Scheme 2: Preparation of a blocked L-deoxyguanosine phosphoramidite



A preferred method of preparing a blocked 2'-deoxy-L-uridine 3'-O-phosphoramidite derivative, U7, that is suitable for use in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotide materials of this invention is outlined in Scheme 3. The reaction of L-arabinose (U1) with cyanamide yields 2'-amino-1,2-oxazoline (U2) which upon treatment with methyl propiolate (U3) affords anhydro-L-uridine (U4). The anhydro derivative U4 is opened with HBr to give the 2'-bromo-nucleoside (U5), which upon catalytic hydrogenation affords 2-deoxy-L-uridine (U6). The 5'-hydroxyl group of U6 is protected as a DMT ether using dimethoxytrityl chloride, and the 3'-hydroxyl is treated with 2-cyanoethyl N,N-

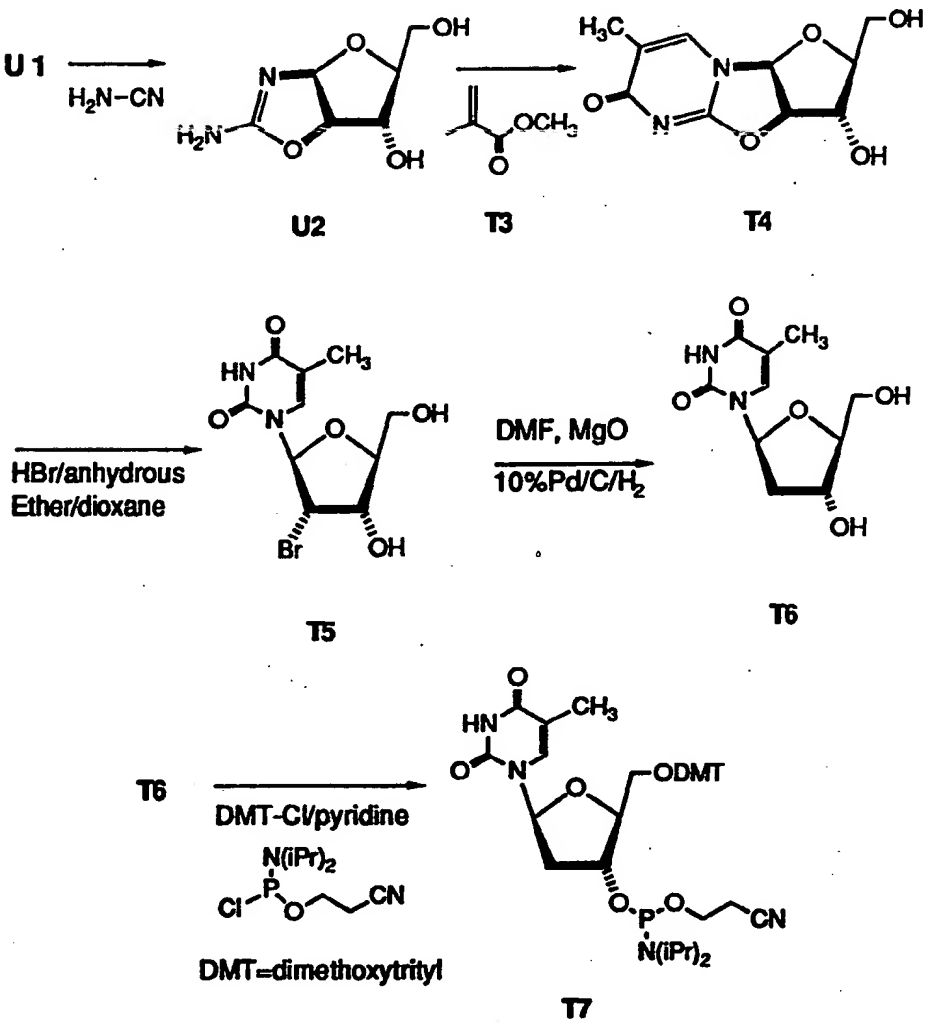
diisopropylchlorophosphoramidite to provide U7, the desired protected 5'-O-dimethoxytrityl-2'-deoxy-L-uridine 3'-O-2-cyanoethylphosphoramidite. U7 is used as an intermediate in the synthesis of L-oligonucleotide sequences of the compositions of Structure IV and Structure V of this invention. Production of such sequences can be done, for example, using an automated oligonucleotide synthesizer using procedures described by the manufacturer for the synthesis and purification of isomeric D-oligonucleotides.

Scheme 3: Preparation of a Blocked L-deoxyuridine phosphoramidite



A preferred method of preparing a blocked L-thymidine 3'-O-phosphoramidite derivative, T7, that is suitable for use in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotide materials of this invention is outlined in Scheme 4. The synthetic method is analogous to the preparation of the 2'-deoxy-L-uridine 3'-O-phosphoramidite derivative, U7, described in Scheme 3 using L-arabinose as a chiral starting material. Accordingly, L-arabinose is treated with cyanamide to provide 2'-amino-1,2-oxazoline (U2) which is then reacted with methyl methacrylate with heating to yield the anhydro-nucleoside (T4). T4 is opened with anhydrous HBr to give 2-bromo-L-thymidine (T5). Upon catalytic hydrogenation T5 affords L-thymidine (T6). The 5'-hydroxyl group of T6 is protected as a DMT ether using dimethoxytrityl chloride, and the 3'-hydroxyl is treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite to provide T7, the desired protected 5'-O-dimethoxytrityl-L-thymidine 3'-O-2-cyanoethylphosphoramidite. T7 is used as an intermediate in the synthesis of L-oligonucleotide sequences of the compositions of Structure IV and Structure V of this invention. Production of such sequences can be done, for example, using an automated oligonucleotide synthesizer using procedures described by the manufacturer for the synthesis and purification of isomeric D-oligonucleotides.

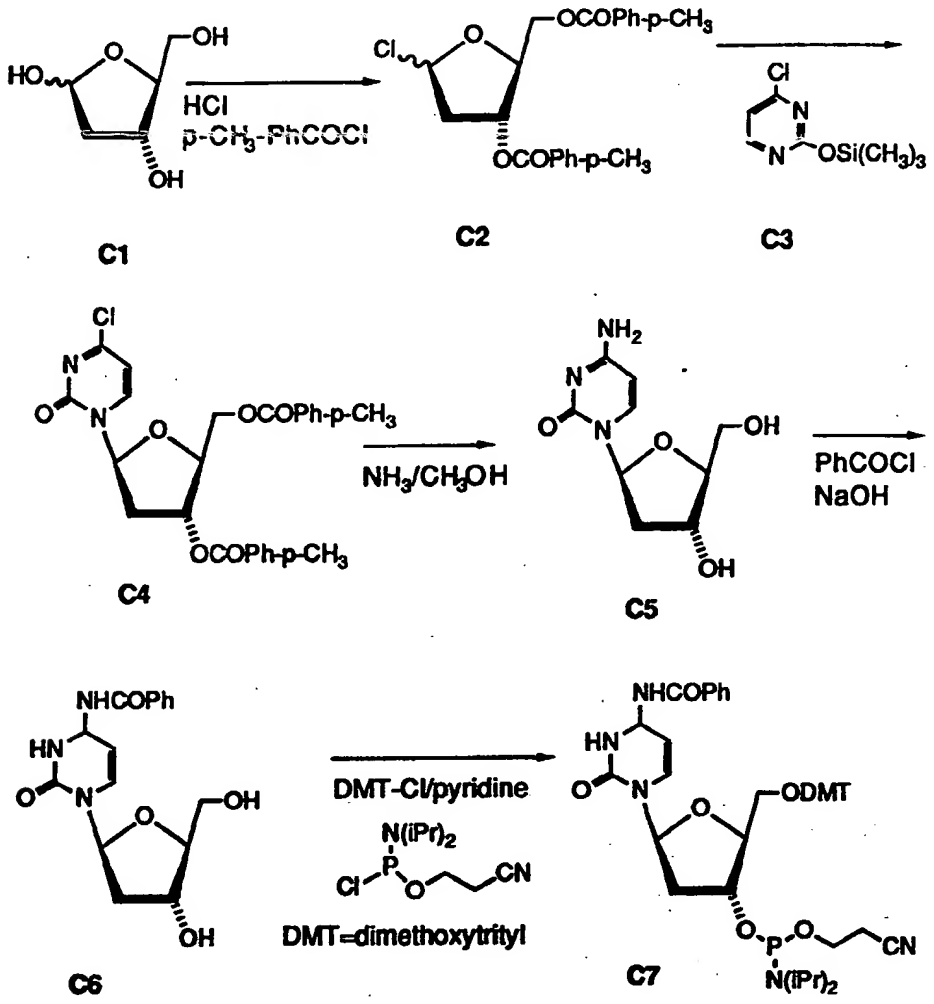
Scheme 4: Preparation of a Blocked L-thymidine phosphoramidite



A preferred method of preparing a blocked 2'-deoxy-L-cytidine 3'-O-phosphoramidite derivative, C7, that is suitable for use in the synthesis of the L-enantiomeric oligo-L-deoxyribonucleotide materials of this invention is outlined in Scheme 5. Thus, L-arabinal, A1, is treated with HCl and toluyl chloride to provide the 3,5-di-O-p-toluyl-2-deoxy-L-ribofuranosyl chloride (C2). Fusion of (C2) with 4-chloro-2-trimethylsiloxypyrimidine (C3) affords the 1-(3,5-di-O-p-toluyl-2-deoxy-L-ribofuranosyl)-4-chloropyrimidin-2-one (C4), which upon

treatment with ammonia in methanol yields 2-deoxy-L-cytidine (C5). The exocyclic amine of C5 is protected by treatment with benzoyl chloride, and the esters at the 3'- and 5'-positions are saponified to regenerate C6 with free 3'- and 5'-hydroxyl groups. The 5'-hydroxyl group of C6 is protected as a DMT ether using dimethoxytrityl chloride, and the 3'-hydroxyl is treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite to provide C7, the desired protected 5'-O-dimethoxytrityl-2'-deoxy-L-cytidine 3'-O-2-cyanoethylphosphoramidite. C7 is used as an intermediate in the synthesis of L-oligonucleotide sequences of the compositions of Structure IV and Structure V of this invention. Production of such sequences can be done, for example, using an automated oligonucleotide synthesizer using procedures described by the manufacturer for the synthesis and purification of isomeric D-oligonucleotides.

Scheme 5: Preparation of a Blocked L-deoxycytidine phosphoramidite



The 2'-deoxy-L-adenosine (A6) is sometimes hereinafter referred to as L-dA; the 2'-deoxy-L-guanosine (G7) is sometimes hereinafter referred to as L-dG; the 2'-deoxy-L-uridine (U6) is sometimes hereinafter referred to as L-dU; the L-thymidine (T6) is sometimes hereinafter referred to as L-T; and the 2'-deoxy-L-cytidine (C5) is sometimes hereinafter referred to as L-dC. Accordingly, as a definition of abbreviated nomenclature for the L-enantiomeric oligonucleotides of this invention, an irrelevant L-enantiomeric

oligonucleotide having a sequence containing, for example, a 2'-deoxy-L-adenosine, L-dA, which is linked by a phosphate diester group to a 2'-deoxy-L-guanosine, L-dG, which in turn is linked by a phosphate diester group to a 2'-deoxy-L-uridine, L-dU, which in turn is linked by a phosphate diester group to an L-thymidine, L-T, which in turn is linked by a phosphate diester group to a 2'-deoxy-L-cytidine, L-dC, would be defined and sometimes referred to as L-d(AGUTC). If the 5'-end of this oligomer is attached to a group R_1 and the 3'-end of this oligomer is attached to a group R_2, then the oligomer would sometimes be referred to as R_1-5'-L-d(AGUTC)-3'-R_2 or sometimes as 5'-R_1-L-d(AGUTC)-R_2-3'. Conversely, if the 3'-end of this oligomer is attached to a group R_1 and the 5'-end of this oligomer is attached to a group R_2, then the oligomer would sometimes be referred to as R_1-3'-L-d(AGUTC)-5'-R_2 or sometimes as 3'-R_1-L-d(AGUTC)-R_2-5'. Reference is made to the L-oligonucleotides of this invention and particularly to the presently preferred oligonucleotides which have the sequences as described hereinbelow using this nomenclature.

The term "residue" is used herein in context with a chemical entity. Said chemical entity comprises, for example, a chelating group, or a linking group, or a protein reactive group, or an immunoreactive group, or an immunoreactive protein, or an antibody, or an antibody fragment, or a cross-linking agent such as a heterobifunctional cross-linking agent, or an L-enantiomeric oligonucleotide, or a spacing group, or an end capping group. The term "residue" is defined as that portion of the chemical entity which exclusively remains when one or more chemical bonds therein when considered as an independent chemical entity, is altered, modified, or replaced to comprise one or more covalent bonds to one or more other chemical entities.

Thus, for example, in one aspect, a "residue of an L-enantiomeric oligonucleotide" in the context of, for example, I and I_i in Structure IV or of cI in Structure V comprises an L-enantiomeric oligonucleotide modified at least for divalent attachment to the residue of another chemical entity, i.e., the residue of said L-enantiomeric oligonucleotide comprises at least a divalent L-enantiomeric oligonucleotidyl sequence. In another aspect, for example, "the residue of a chelating group" in the context of W_1, W_2 or W_3 of Structure V comprises a chelating group which is at least monovalently modified through attachment to the residue of another chemical entity such as, for example, to the residue of a linking group.

In Structure IV above, Z preferably is an antibody or antibody fragment which recognizes and is specific for a tumor associated antigen. In some embodiments, the above-described protein can contain an immunoreactive group covalently bonded thereto through a chemical bond or a linking group derived from the residue of a protein reactive group and the residue of a reactive group on the protein. As used herein, the term "immunoreactive protein" which can be abbreviated by "IRP" also includes an organic compound which is capable of covalently bonding to the protein and which is found in a living organism or is useful in the diagnosis, treatment or genetic engineering of cellular material or living organisms, and which has a capacity for interaction with another component which may be found in biological fluids or associated with cells to be treated such as tumor cells.

The immunoreactive group can be selected from a wide variety of naturally occurring or synthetically prepared materials, including, but not limited to enzymes, amino acids, peptides, polypeptides, proteins, lipoproteins, glycoproteins, lipids, phospholipids,

hormones, growth factors, steroids, vitamins, polysaccharides, viruses, protozoa, fungi, parasites, rickettsia, molds, and components thereof, blood components, tissue and organ components, pharmaceuticals, haptens, lectins, toxins, nucleic acids (including oligonucleotides), antibodies (monoclonal and polyclonal), anti-antibodies, antibody fragments, antigenic materials (including proteins and carbohydrates), avidin and derivatives thereof, biotin and derivatives thereof, and others known to one skilled in the art. In addition, an immunoreactive group can be any substance which when presented to an immunocompetent host will result in the production of a specific antibody capable of binding with that substance, or the antibody so produced, which participates in an antigen-antibody reaction.

Preferred immunoreactive groups are antibodies and various immunoreactive fragments thereof, as long as they contain at least one reactive site for reaction with a protein reactive group as described herein on the residue of the L-enantiomeric oligonucleotide or with linking groups as described herein. That site can be inherent to the immunoreactive species or it can be introduced through appropriate chemical modification of the immunoreactive species. In addition to antibodies produced by the techniques outlined above, other antibodies and proteins produced by the techniques of molecular biology are specifically included.

Preferably, the immunoreactive group does not bind to the residue of an L-enantiomeric oligonucleotide in structure IV so as to inhibit the binding of the L-enantiomeric oligonucleotide to a complementary sequenced L-enantiomeric oligonucleotide of structure V.

As used herein, the term "antibody fragment" refers to an immunoreactive material which comprises a residue of an antibody, which antibody characteristically

exhibits an affinity for binding to an antigen. The term affinity for binding to an antigen, as used herein, refers to the thermodynamic expression of the strength of interaction or binding between an antibody combining site and an antigenic determinant and, thus, of the stereochemical compatibility between them. As such, it is the expression of the equilibrium or association constant for the antibody-antigen interaction. The term "affinity" as used herein also refers to the thermodynamic expression of the strength of interaction or binding between a ligand and a receptor and, thus, of the stereochemical compatibility between them. As such, it is the expression of the equilibrium or association constant for the ligand-receptor interaction.

With respect to the affinity of binding of an antibody to an antigen, antibody fragments exhibit a percentage of said affinity for binding to said antigen, that percentage being in the range of 0.001 per cent to 1,000 per cent, preferably 0.01 per cent to 1,000 per cent, more preferably 0.1 per cent to 1,000 per cent, and most preferably 1.0 per cent to 100 per cent, of the relative affinity of said antibody for binding to said antigen.

An antibody fragment can be produced from an antibody by a chemical reaction comprising one or more chemical bond cleaving reactions; by a chemical reaction comprising of one or more chemical bond forming reactions employing as reactants one or more chemical components selected from a group comprising amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, protein reactive groups as defined herein, and antibody fragments such as are produced as described herein and by a molecular biological process, a bacterial process, or by a process comprising or resulting from the genetic engineering of antibody genes.

An antibody fragment can be derived from an antibody by a chemical reaction comprising one or more of the following reactions:

(a) cleavage of one or more chemical bonds of which an antibody is comprised, said bonds being selected from, for example, carbon-nitrogen bonds, sulfur-sulfur bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and wherein the method of said cleavage is selected from:

(i) a catalysed chemical reaction comprising the action of a biochemical catalyst such as an enzyme such as papain or pepsin which enzymes to those skilled in the art are known to produce antibody fragments commonly referred to as Fab and Fab'2, respectively;

(ii) a catalysed chemical reaction comprising the action of an electrophilic chemical catalyst such as a hydronium ion which, for example, favorably occurs at a pH equal to or less than 7;

(iii) a catalysed chemical reaction comprising the action of a nucleophilic catalyst such as a hydroxide ion which, for example, favorably occurs at a pH equal to or greater than 7;

(iv) a chemical reaction comprising a substitution reaction employing a reagent such which is consumed in a stoichiometric manner such as, for example, a substitution reaction at a sulfur atom of a disulfide bond by a reagent comprising a sulfhydryl group (comprising a -SH group) or an anionic sulfide group (comprising an $-S^-$ group in the form of a salt such as a $-S^- Na^+$ group);

(v) a chemical reaction comprising a reduction reaction such as, for example, the reduction of a disulfide bond; and

(vi) a chemical reaction comprising an oxidation reaction such as the oxidation of a carbon-oxygen bond of a hydroxyl group or the oxidation of a carbon-carbon

bond of a vicinal diol group such as occurs in a carbohydrate moiety; or

(b) formation of one or more chemical bonds between one or more reactants, such as formation of one or more covalent bonds selected from, for example, carbon-nitrogen bonds (such as, for example, amide bonds, amine bonds, hydrazone bonds, imine bonds, and thiourea bonds), sulfur-sulfur bonds such as disulfide bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and employing as reactants in said chemical bond formation one or more reagents comprising amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, protein reactive groups as defined herein, and antibody fragments such as are produced as described in (a), above; or

(c) an antibody fragment can be derived by formation of one or more non-covalent bonds between one or more reactants. Such non-covalent bonds comprise hydrophobic interactions such as occur in an aqueous medium between chemical species that independently comprise mutually accessible regions of low polarity such as regions comprising aliphatic and carbocyclic groups, and of hydrogen bond interactions such as occur in the binding of an oligonucleotide with a complementary oligonucleotide; or

(d) an antibody fragment can be produced as a result of the methods of molecular biology or by genetic engineering of antibody genes, for example, in the genetic engineering of a single chain immunoreactive group or a Fv fragment.

An antibody fragment can be produced as a result a combination of one or more of the above methods.

In certain embodiments, the immunoreactive group can be an enzyme which has a reactive group for attachment to the residue of an L-enantiomeric

oligonucleotide, I, by means of a linking group L_Z. Representative enzymes include, but are not limited to, aspartate aminotransaminase, alanine aminotransaminase, lactate dehydrogenase, creatine phosphokinase, gamma glutamyl transferase, alkaline acid phosphatase, prostatic acid phosphatase, horseradish peroxidase and various esterases.

If desired, the immunoreactive group can be modified or chemically altered to provide a reactive group for use in the attachment to the residue of the L-enantiomeric oligonucleotide, I, through a linking group as described below by techniques known to those skilled in the art. Such techniques include the use of linking moieties and chemical modification such as described in WO-A-89/02931 and WO-A-89/2932, which are directed to modification of oligonucleotides, and U.S. Patent No. 4,719,182.

Two highly preferred uses for the compositions of this invention are for the diagnostic imaging of tumors and the radiological treatment of tumors. Preferred immunological groups therefore include antibodies to tumor-associated antigens. An antibody is sometimes hereinafter referred to as Ab. Specific non-limiting examples of antibodies include B72.3 and related antibodies (described in U.S. Patent Nos. 4,522,918 and 4,612,282) which recognize colorectal tumors; 9.2.27 and related anti-melanoma antibodies; D612 and related antibodies which recognize colorectal tumors; UJ13A and related antibodies which recognize small cell lung carcinomas; NRLU-10, NRCO-02 and related antibodies which recognize small cell lung carcinomas and colorectal tumors (Pan-carcinoma); 7E11C5 and related antibodies which recognize prostate tumors; CC49 and related antibodies which recognize colorectal tumors; TNT and related antibodies which recognize necrotic tissue; PR1A3 and related antibodies which recognize

colon carcinoma; ING-1 and related antibodies, which are described in International Patent Publication WO-A-90/02569; B174, C174 and related antibodies which recognize squamous cell carcinomas; B43 and related antibodies which are reactive with certain lymphomas and leukemias; and anti-HLB and related monoclonal antibodies. An especially preferred antibody is ING-1.

Referring to structure IV again, L_Z and L_Q are independently a chemical bond or the residue of a linking group. The phrase "residue of a linking group" as used herein refers to a moiety that remains, results, or is derived from the reaction of a protein reactive group with a reactive site on the protein. The phrase "protein reactive group" as used herein refers to any group which can react with a functional group typically found on a protein. However, it is specifically contemplated that a protein reactive groups can also react with a functional group typically found on a non-protein biomolecule. Thus a linking group useful in the practice of this invention derives from a group which can react with any biological molecule containing an immunoreactive group, whether or not the biological molecule is a protein, to form a linking group between the immunoreactive group and the L-enantiomeric oligonucleotide containing species as described below.

Preferred linking groups are derived from protein reactive groups selected from but not limited to:

(1) A group that will react directly with amine, alcohol, or sulfhydryl groups on the protein or biological molecule containing the immunoreactive group, for example, active halogen containing groups including, for example, chloromethylphenyl groups and chloroacetyl [$ClCH_2C(=O)-$] groups, activated 2-(leaving group substituted)-ethylsulfonyl and ethylcarbonyl groups such as 2-chloroethylsulfonyl and 2-chloroethylcarbonyl; vinylsulfonyl; vinylcarbonyl; epoxy; isocyanato;

isothiocyanato; aldehyde; aziridine; succinimidoxycarbonyl; activated acyl groups such as carboxylic acid halides; mixed anhydrides and the like; and other groups known to be useful in conventional photographic gelatin hardening agents;

(2) A group that can react readily with modified proteins or biological molecules containing the immunoreactive group, i.e., proteins or biological molecules containing the immunoreactive group modified to contain reactive groups such as those mentioned in (1) above, for example, by oxidation of a group such as a hydroxyl group of a protein to an aldehyde or to a carboxylic acid, in which case the "linking group" can be derived from protein reactive groups selected from amino, alkylamino, arylamino, hydrazino, alkylhydrazino, arylhydrazino, carbazido, semicarbazido, thiocarbazido, thiosemicarbazido, sulfhydryl, sulfhydrylalkyl, sulfhydrylaryl, hydroxy, carboxy, carboxyalkyl and carboxyaryl. The alkyl portions of said linking groups can contain from 1 to about 20 carbon atoms. The aryl portions of said linking groups can contain from about 6 to about 20 carbon atoms;

(3) A group that can be linked to the protein or biological molecule containing the immunoreactive group, or to the modified protein as noted in (1) and (2) above by use of a crosslinking agent. The residues of certain useful crosslinking agents, such as, for example, homobifunctional and heterobifunctional gelatin hardeners, bisepoxides, and bisisocyanates can become a part of, i.e., a linking group in, the protein-(L-enantiomeric oligonucleotide-containing species) conjugate during the crosslinking reaction. Other useful crosslinking agents, however, can facilitate the crosslinking, for example, as consumable catalysts, and are not present in the final conjugate. Examples of such crosslinking agents are carbodiimide and

carbamoylonium crosslinking agents as disclosed in U.S. Patent No. 4,421,847 and the ethers of U.S. Patent No. 4,877,724. With these crosslinking agents, one of the reactants, such as the immunoreactive group, must have a carboxyl group and the other, such as the L-enantiomeric oligonucleotide-containing species, must have a reactive amine, alcohol, or sulfhydryl group. In amide bond formation, the crosslinking agent first reacts selectively with the carboxyl group, then is split out during reaction of the thus "activated" carboxyl group with an amine to form an amide linkage between the protein and L-enantiomeric oligonucleotide containing species, thus covalently bonding the two moieties. An advantage of this approach is that crosslinking of like molecules, e.g., proteins with proteins or L-enantiomeric oligonucleotide containing species with themselves is avoided, whereas the reaction of, for example, homo-bifunctional crosslinking agents is nonselective and unwanted crosslinked molecules are obtained.

Preferred useful linking groups are derived from various heterobifunctional cross-linking reagents such as those listed in the Pierce Chemical Company Immunotechnology Catalog - Protein Modification Section, (1991 and 1992).

Useful non-limiting examples of such reagents include:

Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate;
Sulfo-SIAB	Sulfosuccinimidyl (4-iodoacetyl)aminobenzoate;

Sulfo-SMPB	Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate;
2-IT	2-Iminothiolane; and
SATA	N-Succinimidyl S-acetylthioacetate.

In addition to the foregoing description, the linking groups, in whole or in part, can also comprise and be derived from nucleotides and residues of nucleotides, both naturally occurring and modified. Particularly useful, non-limiting reagents for incorporation of modified nucleotide moieties containing reactive functional groups, such as amine and sulfhydryl groups, into an L-enantiomeric oligonucleotide sequence of this invention are commercially available from, for example, Clonetech Laboratories Inc. (Palo Alto California) and include Uni-Link AminoModifier (Catalog # 5190), Biotin-ON phosphoramidite (Catalog # 5191), N-MNT-C6-AminoModifier (Catalog # 5202), AminoModifier II (Catalog # 5203), DMT-C6-3'Amine-ON (Catalog # 5222), C6-ThiolModifier (Catalog # 5211), and the like. In one aspect, linking groups of this invention are derived from the reaction of a reactive functional group such as an amine or sulfhydryl group as are available in the above Clonetech reagents, one or more of which has been incorporated into an L-enantiomeric oligonucleotide sequence of this invention, with, for example, one or more of the previously described protein reactive groups such as heterobifunctional protein reactive groups, one or more of which has been incorporated into an immunoreagent of this invention.

Referring to Structure IV again, I and I_i each independently comprise an L-enantiomeric oligonucleotide of a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous

sequence contains one or more members of a family of homologous contiguous sequences; wherein the individual homologs of said family comprise from 12 to about 30 L-enantiomeric nucleotide units; wherein the homologs of said family of sequences, both individually or as a set of homologous sequences being hereinafter sometimes referred to as "the Sequence"; and wherein any contiguous sequence of six or more L-enantiomeric nucleotide units does not hybridize with any other contiguous sequence of six or more contiguous L-enantiomeric nucleotide units anywhere in structure IV. Members of the set of homologous contiguous sequences which comprise "the Sequence" can be found in both the sequence I and the sequence I_i, and at least one such sequence is common to both I and I_i.

The L-enantiomeric oligonucleotide sequence of I and I_i in Structure IV can comprise L-DNA, L-RNA, purine and pyrimidine base-modified L-DNA or L-RNA, backbone-modified L-DNA or L-RNA such as methyl phosphonate or thiophosphonate or carbohydrate modified L-DNA or L-RNA analogs, whole or partially modified, or combinations thereof as long as a complementary L-enantiomeric oligonucleotide sequence incorporated into the radioactive targeting moiety of Structure V described below can hybridize to said L-enantiomeric oligonucleotide sequence to form a hybrid which exhibits a Tm (melting temperature) greater than about 37 ^{o}C. Preferred L-enantiomeric oligonucleotides are non-base-modified and non-backbone-modified L-DNA and L-RNA, more preferred are L-DNA comprising L-dA, L-T, L-dG, L-dU and L-dC L-enantiomeric nucleotide units. Currently especially preferred L-enantiomeric oligonucleotides are L-DNA comprising L-dA, L-T, L-dG, and L-dC L-enantiomeric nucleotide units.

In a preferred embodiment, the L-enantiomeric oligonucleotide sequence I and I_i can comprise double

stranded L-DNA or L-RNA. That is, the L-enantiomeric oligonucleotide sequence may comprises complementary L-DNA or L-RNA which forms a double helix molecule. The complementary L-enantiomeric oligonucleotide sequence incorporated into the radioactive targeting moiety, composed of L-DNA or L-RNA, then hybridizes to one or the other of the strands of the double stranded L-DNA or L-RNA comprising I and I_i. In this way, the complementary L-enantiomeric oligonucleotide sequence incorporated into the radioactive targeting moiety interacts with the duplex L-DNA or L-RNA of I and I_i in such a way as to form triplex (triple helix) L-DNA, triplex L-RNA, or a triplex L-DNA:L-RNA hybrid.

Preferred non-limiting examples of L-enantiomeric oligonucleotide sequences comprising the "Sequence" are shown below. The following sequences comprise a set of homologous L-enantiomeric oligonucleotide sequences which when considered individually or in any combination comprise a set herein defined as the "Sequence":

(i) L-d(TTATGGACGGAG) (SEQ ID NO:1);
(ii) L-d(TTATGGACGGAGA) (SEQ ID NO:2);
(iii) L-d(TTATGGACGGAGAA) (SEQ ID NO:3);
(iv) L-d(TTATGGACGGAGAAG) (SEQ ID NO:4);
(v) L-d(TTATGGACGGAGAAGC) (SEQ ID NO:5);
(vi) L-d(TTATGGACGGAGAAGCT) (SEQ ID NO:6);
(vii) L-d(TTATGGACGGAGAAGCTA) (SEQ ID NO:7); and
(viii) L-d(TTATGGACGGAGAAGCTAA) (SEQ ID NO:8).

Of course, sequence (viii) contains sequence (vii) which contains sequence (vi) which contains sequence (v), and so on. Thus, in structure IV, if I contains, for example sequence (iii), and an I_i contains, for example, sequence (v), then both I and I_i contain at least sequence (iii). Another I_i in structure IV can contain (viii), in which case it would also contain (i) through (vii) as well as (viii). In this case, all three sequences would contain at least (iii) [as well as

(i) and (ii)], and the two I_1's would contain at least (v) [as well as (i) through (iv)]. In this regard, an L-enantiomeric oligonucleotide that comprises a contiguous sequence of L-enantiomeric nucleotides, which sequence being complementary to at least sequence (i), would hybridize to all sequences (i) through (viii) as would any member of a set of contiguous complementary sequences, the individual members of which comprise the sequence complementary to any of (i) through (viii). Such a set of contiguous complementary sequences can comprise cI as will be described below.

Another set of preferred homologous L-enantiomeric oligonucleotide sequences comprising the "Sequence" is:

(ix) L-d(CGGAGAAGCTAA) (SEQ ID NO:9);
(x) L-d(ACGGAGAAGCTAA) (SEQ ID NO:10);
(xi) L-d(GACGGAGAAGCTAA) (SEQ ID NO:11);
(xii) L-d(GGACGGAGAAGCTAA) (SEQ ID NO:12);
(xiii) L-d(TGGACGGAGAAGCTAA) (SEQ ID NO:13);
(xiv) L-d(ATGGACGGAGAAGCTAA) (SEQ ID NO:14);
(xv) L-d(TATGGACGGAGAAGCTAA) (SEQ ID NO:15); and
(xvi) L-d(TTATGGACGGAGAAGCTAA) (SEQ ID NO:8).

An especially preferred sequence is: L-d(TTATGGACGGAGAAGCTAA) (SEQ ID NO:8).

Two or more of the L-enantiomeric oligonucleotide sequences of this invention can be tandemly linked by means of chemical bonds, by linking groups such as described above, or by spacing groups as described below. The sequential order of L-enantiomeric nucleotides in the L-enantiomeric oligonucleotide sequences of this invention can be from the 5' to the 3' end or from the 3' to the 5' end. Attachment of the L-enantiomeric oligonucleotide sequences of this invention via linking groups as described above to the immune reactive group as described above can be accomplished via 3' or via 5' sites or via derivatives attached to 3' or 5' sites of the L-enantiomeric oligonucleotide.

As discussed above, the "Sequence" may also be composed of a double stranded L-DNA or L-RNA. That is, the "Sequence" may consist of complementary L-enantiomeric oligonucleotides which non-covalently interact to form double stranded L-DNA or L-RNA. Attachment of this double stranded nucleic acid to the immune reactive group as described above can be accomplished via 3' or via 5' sites or via derivatives attached to 3' or 5' sites of the L-enantiomeric oligonucleotide.

Referring to structure IV again, Q_I is a spacing group which separates and links two or more L-enantiomeric oligonucleotide sequences of this invention. Q_I can comprise a linking group as defined above, alone, or in combination with an L-enantiomeric nucleotide or an L-enantiomeric oligonucleotide comprising 2 to about 20 L-enantiomeric nucleotide units, the sequence of which is not self-associating or such that contiguous sequences of six or more L-enantiomeric nucleotide units therein do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure IV. Q_I can also comprise a residue of an amino acid group, a peptide group, or a poly(alkylene oxide) group such as a poly(ethylene glycol) group. It is contemplated that each spacing group can be linked to from two to about six L-enantiomeric oligonucleotide sequences, at least two of which contain the Sequence of this invention. Preferably, the spacing group is linked to two L-enantiomeric oligonucleotide sequences each of which contains the Sequence of this invention. Preferably, the spacing group is an L-enantiomeric oligonucleotide sequence.

Non limiting examples of preferred spacing groups are L-enantiomeric oligonucleotides comprising the following sequences:

L-d(ACT);
L-d(ACTC);
L-d(ACTCT);
L-d(CTC);
L-d(TCTC); and
L-d(CTCTC).

An especially preferred spacing group is an L-enantiomeric oligonucleotide: L-d(ACTCTC).

Of course, the condition described above, i.e., that the L-enantiomeric oligonucleotide sequence of this invention comprises a non-self associating sequence, still applies when considering the selection of L-enantiomeric oligonucleotide spacing groups linked in combination with the L-enantiomeric oligonucleotide Sequence groups.

In structure IV, a is from 0 to about 6, preferably an integer from 1 to about 6, more preferably one to about 4, and most preferably one or two.

In structure IV, p is an integer from 1 to 10, preferably 1 to about 6, and more preferably 1 to 3. It is also contemplated that mixtures of immunoreactive proteins comprising mixtures of Z modified as defined in structure IV together with Z not so modified will also be useful in this invention. In this case, the bulk mixture properties of "p" of such mixtures would comprise fractional values from about zero to about 10. Preferably, the bulk p values would be from about 0.1 to about 10.0, more preferably from about 0.2 to about 5.0, and most preferably from about 0.4 to about 3.

In structure IV, E is an end capping group. E is preferably an L-enantiomeric nucleotide group or a group of one or more D-oligonucleotides that is modified so as to reduce or prevent the action of exonuclease enzymatic activity on the D-enantiomeric oligonucleotide sequence therein. E can be a 3'- or 5'-phosphate-linked ribose group containing one or more substituents such as an

alkyl group of 1 to about 10 carbon atoms. E can be a 5'- or 3'-ether group such as an alkyl ether, an aryl ether, an aralkyl ether, a substituted aryl ether or an aralkyl ether wherein the alkyl groups contain from 1 to about 10 carbon atoms and the aryl groups contain from 6 to 10 carbon atoms, and wherein the alkyl or aryl groups may contain oxygen, nitrogen or sulfur atoms or be substituted by alkyl or aryl groups containing oxygen, nitrogen or sulfur atoms. E can be a 5'-O- or 3'-O-phosphate ester group such as an alkyl ester, an aryl ester, an aralkyl ester, a substituted aryl ester or an aralkyl ester wherein the alkyl groups contain from 1 to about 10 carbon atoms and the aryl groups contain from 6 to 10 carbon atoms, and wherein the alkyl or aryl groups may contain oxygen, nitrogen or sulfur atoms or be substituted by alkyl or aryl groups containing oxygen, nitrogen or sulfur atoms. E can be a poly(alkylene oxidyl) group on the ribosyl moiety, preferably at the 5'- or 3'- position, either as an ether group or linked by a phosphate ester to the 5'- or 3'-oxygen of the L-ribosyl group. The poly(alkylene oxidyl) group can be, for example, a poly(ethylene oxidyl) or poly(propylene oxidyl) or a poly(propylene oxidyl-co-ethylene oxidyl) group, each polymer containing from 2 to about 100 repeating units. A phosphate ester comprising such entities is also useful, as well as a phosphate ester or modified ribose comprising elements of a suitable linking group as defined above. E can also comprise a Z or it can be attached to Q_I by elements of L_Z as defined above to form a cyclic structure. E can also comprise compounds with a two carbon-one nitrogen atom internucleoside linkage. Preferably, E comprises a poly(alkylene glycol) phosphate diester. With respect to E, the poly(alkylene glycol) moiety can have from 2 to about 100 repeating units. Preferably, the poly(alkylene glycol) is a poly(ethylene glycol). A

currently preferred poly(alkylene glycol) phosphate diester is a tetra(ethylene glycol) phosphate diester, hereinafter sometimes referred to as a "Teg" or "Teg unit". Such poly(alkylene glycol) phosphate diesters can be linked in tandem to each other to form a dimer phosphate ester sequence, a trimer sequence, a tetramer sequence, and so forth. One or two such units is preferred. Such units can also be attached to residues of Q_I, L_Z, L_Q, I, I_i or Z described above. A preferred end group E comprises a Teg unit linked by a phosphate ester bond to an L-enantiomeric nucleotide such as T. Other preferred end capping units for an L-DNA sequence comprise a residue of L-dA, L-dG, L-T, L-dC, and L-dU or an oligonucleotide sequence comprised therefrom.

In the context of this invention, the term "modified nucleotide moiety" is intended to mean a chemical entity which comprises one or more chemical groups that are analogous to one or more portions of a naturally occurring D-enantiomeric nucleotide or of a residue of a naturally occurring D-enantiomeric nucleotide. A "modified D-enantiomeric nucleotide moiety" comprises that chemical entity which exclusively remains when one or more chemical bonds, of which said naturally occurring D-enantiomeric nucleotide is otherwise comprised when considered as an independent chemical entity, is altered, modified, or replaced to comprise one or more covalent bonds to one or more other chemical entities, or comprises that chemical entity which exclusively remains after removal or deletion of a portion, such as, for example, a purine or pyrimidine base portion, a hydroxyl portion, a ribose portion, and the like or combinations thereof, of the naturally occurring D-enantiomeric nucleotide in one location simultaneously with said replacement of another portion of said L-enantiomeric nucleotide. Particularly useful, non-limiting examples of modified D-enantiomeric

nucleotide moieties comprise reactive functional groups, such as amine and sulfhydryl groups. They can be commercially available such as, for example, those modified D-enantiomeric nucleotide moieties and precursors thereto which are available from Clonetech Laboratories Inc. (Palo Alto, California). Said modified D-enantiomeric nucleotide moieties and precursors thereto include Uni-Link AminoModifier (Catalog #5190), Biotin-ON phosphoramidite (Catalog #5191), N-MNT-C6-AminoModifier (Catalog #5202), AminoModifier II (Catalog #5203), DMT-C6-3'Amine-ON (Catalog #5222), C6-ThiolModifier (Catalog #5211), and the like. One or more of said moieties can be incorporated into an L-enantiomeric oligonucleotide sequence comprising this invention. In one aspect, linking groups of this invention are derived from the reaction of a reactive functional group such as an amine or sulfhydryl group as are available in the above Clonetech reagents, one or more of which has been incorporated into an L-enantiomeric oligonucleotide sequence of this invention, with, for example, one or more of the previously described protein reactive groups such as heterobifunctional protein reactive groups, one or more of which has been incorporated into an immunoreagent of this invention.

A "modified D-enantiomeric nucleotide moiety" can comprise a D-enantiomeric nucleotide moiety that is modified so as to reduce or prevent the action of exonuclease enzymatic activity on the D-enantiomeric oligonucleotide sequence. It can be a 3'- or 5'-phosphate linked ribose or a 3'- or 5'-phosphate linked 2'-deoxyribose group containing one or more substituents such as an alkyl group of 1 to about 10 carbon atoms, or an ether group such as alkyl or aryl or aralkyl or substituted aryl or aralkyl ether wherein the alkyl groups contain from 1 to about 10 carbon atoms and such

alkyl or aryl groups may contain or be substituted by substituents containing oxygen, nitrogen or sulfur atoms, or a poly(alkylene oxidyl) group, preferably at the 5'- or 3'-ribose position, respectively, or elsewhere on the ribose group, which substituent will reduce or prevent the action of exonuclease enzymatic activity. A "modified D-enantiomeric nucleotide moiety" comprising a phosphate ester comprising said substituents is also useful, as well as a phosphate ester or modified ribose comprising elements of a suitable linking group as defined above. A "modified D-enantiomeric nucleotide moiety" can also comprise a residue of Z or it can be attached to Q_I by elements of L_Z as defined above to form a cyclic structure. A "modified D-enantiomeric nucleotide moiety" can also comprise compounds with a two carbon-one nitrogen atom internucleoside linkage.

Preferably, a "modified D-enantiomeric nucleotide moiety" comprises a poly(alkylene glycol) phosphate diester. With respect to said "modified D-enantiomeric nucleotide moiety", the poly(alkylene glycol) moiety can have from 2 to about 100 repeating units. Preferably, the poly(alkylene glycol) is a poly(ethylene glycol). A currently preferred poly(alkylene glycol) phosphate diester is a tetra(ethylene glycol) phosphate diester, hereinafter sometimes referred to as a "Teg" or "Teg unit". Such poly(alkylene glycol) phosphate diesters can be linked in tandem to each other to form a dimer phosphate ester sequence, a trimer sequence, a tetramer sequence, and so forth. One or two such units is preferred. Such units can also be attached to residues of Q_I, L_Z, L_Q, I, I_i or Z described herein. A preferred "modified D-enantiomeric nucleotide moiety" comprises a Teg unit linked by a phosphate ester bond to a D-enantiomeric nucleotide such as L-T.

Referring to the radioactive targeting immunoreagent described in Structure V, preferred non-limiting examples of a set of L-enantiomeric oligonucleotides, cI, which are complementary to the members of the set of L-enantiomeric oligonucleotides comprising the "Sequence" of I in structure IV include the L-DNA L-enantiomeric oligonucleotides:

(xvii) L-d(TTAGCTTCTCCG) (SEQ ID NO:16);
(xviii) L-d(TTAGCTTCTCCGT) (SEQ ID NO:17);
(xix) L-d(TTAGCTTCTCCGTC) (SEQ ID NO:18);
(xx) L-d(TTAGCTTCTCCGTCC) (SEQ ID NO:19);
(xxi) L-d(TTAGCTTCTCCGTCCA) (SEQ ID NO:20);
(xxii) L-d(TTAGCTTCTCCGTCCAT) (SEQ ID NO:21);
(xxiii) L-d(TTAGCTTCTCCGTCCATA) (SEQ ID NO:22);
and
(xxiv) L-d(TTAGCTTCTCCGTCCATAA) (SEQ ID NO:23)

Another set of preferred homologous L-enantiomeric oligonucleotide sequences which can comprise a set complementary to the "Sequence" of structure IV is:

(xxv) L-d(CTCCGTCCATAA) (SEQ ID NO:24);
(xxvi) L-d(TCTCCGTCCATAA) (SEQ ID NO:25);
(xxvii) L-d(TTCTCCGTCCATAA) (SEQ ID NO:26);
(xxviii) L-d(CTTCTCCGTCCATAA) (SEQ ID NO:27);
(xxix) L-d(GCTTCTCCGTCCATAA) (SEQ ID NO:28);
(xxx) L-d(AGCTTCTCCGTCCATAA) (SEQ ID NO:29);
(xxxi) L-d(TAGCTTCTCCGTCCATAA) (SEQ ID NO:30);
(xxxii) L-d(TTAGCTTCTCCGTCCATAA) (SEQ ID NO:23)

An especially preferred complementary sequence comprises:

L-d(TTAGCTTCTCCGTCCATAA) (SEQ ID NO: 23)

The complementarity of the above cI L-enantiomeric oligonucleotide sequences of structure V with respect to the previously listed "I" L-enantiomeric oligonucleotide sequences of structure IV depends on the relative orientation of each, i.e., whether the sequences of I and of cI are constructed from 5' to 3' or 3' to 5', or

vice versa, respectively. Preferably, the Tm of a hybridized complex formed between the respective I and cI sequences is greater than 37°C.

In a preferred embodiment, the L-enantiomeric oligonucleotide sequence cI can comprise double stranded L-DNA or L-RNA. That is, the L-enantiomeric oligonucleotide sequence may comprise complementary L-DNA or L-RNA which forms a double helix molecule. The complementary L-enantiomeric oligonucleotide sequence incorporated into the non-radioactive targeting immunoreagent composed of L-DNA or L-RNA then hybridizes to one or the other of the strands of the double stranded L-DNA or L-RNA comprising cI. In this way, the complementary L-enantiomeric oligonucleotide sequence incorporated into the non-radioactive targeting immunoreagent interacts with the duplex L-DNA or L-RNA of cI in such a way as to form triplex (triple helix) L-DNA, triplex L-RNA, or a triplex L-DNA:L-RNA hybrid.

Q_{cI} in structure V is a spacing group; Q_{cI} can be selected from Q_I as described for structure IV. Preferably, Q_{cI} comprises an L-enantiomeric oligonucleotide contiguous sequence of from 2 to about 30 L-enantiomeric nucleotides wherein the sequence of which is not self-associating and wherein a contiguous sequence of six or more L-enantiomeric nucleotide units does not hybridize with any other contiguous sequence of six or more contiguous L-enantiomeric nucleotide units anywhere in structure V. Q_{cI} can also comprise an L-enantiomeric oligonucleotide, preferably a sequence such as (xxvii) to (xxxii) above which is complementary to the "Sequence" in structure IV. Preferably, Q_{cI} comprises one or two such sequences.

L_1, L_2, and L_3 in structure V are independently a chemical bond, preferably a phosphate ester bond, or a linking group which are defined as L_Z and L_Q in the

above structure V. L_1, L_2, and L_3 can also independently comprise components of QcI.

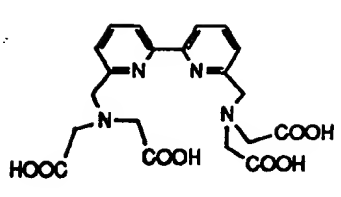
W_1, W_2, and W_3 in structure V are residues of chelating groups. The chelating groups of this invention can comprise the residue of one or more of a wide variety of chelating agents that can have a radionuclide associated therewith. As is well known, a chelating agent is a compound containing donor atoms that can combine by coordinate bonding with a metal atom to form a cyclic structure called a chelation complex or chelate. This class of compounds is described in the Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 5, 339-368.

The residues of suitable chelating agents can be independently selected from polyphosphates, such as sodium tripolyphosphate and hexametaphosphoric acid; aminocarboxylic acids, such as ethylenediaminetetraacetic acid, N-(2-hydroxyethyl)ethylene-diaminetriacetic acid, nitrilotriacetic acid, N,N-di(2-hydroxyethyl)glycine, ethylenebis(hydroxyphenylglycine) and diethylenetriamine pentacetic acid; 1,3-diketones, such as acetylacetone, trifluoroacetylacetone, and thenoyltrifluoroacetone; hydroxycarboxylic acids, such as tartaric acid, citric acid, gluconic acid, and 5-sulfosalicylic acid; polyamines, such as ethylenediamine, diethylenetriamine, triethylenetetramine, and triaminotriethylamine; aminoalcohols, such as triethanolamine and N-(2-hydroxyethyl)ethylenediamine; aromatic heterocyclic bases, such as 2,2'-dipyridyl, 2,2'-diimidazole, dipicoline amine and 1,10-phenanthroline; phenols, such as salicylaldehyde, disulfopyrocatechol, and chromotropic acid; aminophenols, such as 8-hydroxyquinoline and oximesulfonic acid; oximes, such as dimethylglyoxime and salicylaldoxime; peptides containing proximal chelating functionality such as

polycysteine, polyhistidine, polyaspartic acid, polyglutamic acid, or combinations of such amino acids; Schiff bases, such as disalicylaldehyde 1,2-propylenediimine; tetrapyrroles, such as tetraphenylporphin and phthalocyanine; sulfur compounds, such as toluenedithiol, meso-2,3-dimercaptosuccinic acid, dimercaptopropanol, thioglycolic acid, potassium ethyl xanthate, sodium diethyldithiocarbamate, dithizone, diethyl dithiophosphoric acid, and thiourea; synthetic macrocylic compounds, such as dibenzo[18]crown-6, (CH_3)6-[14]-4,11-diene-N4, and (2.2.2-cryptate); and phosphonic acids, such as nitrilotrimethylene-phosphonic acid, ethylenediaminetetra(methylenephosphonic acid), and hydroxyethylidenediphosphonic acid, or combinations of two or more of the above agents.

Preferred residues of chelating agents contain polycarboxylic acid groups and include: ethylenediamine-N, N, N',N'-tetraacetic acid (EDTA); N,N,N',N",N"-diethylene-triaminepentaacetic acid (DTPA); 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA); 1,4,7,10-tetraazacyclododecane-N,N',N"-triacetic acid (DO3A); 1-oxa-4,7,10-triazacyclododecane-N,N',N"-triacetic acid (OTTA); trans(1,2)-cyclohexanodiethylenetriamine pentaacetic acid (CDTPA);

Preferred residues of chelating agents contain polycarboxylic acid groups and include the following:



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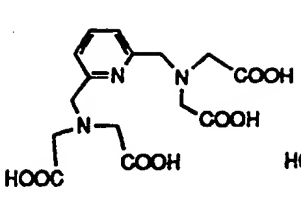

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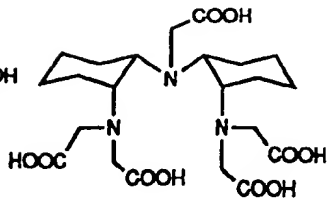

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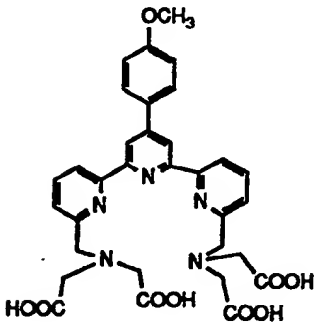

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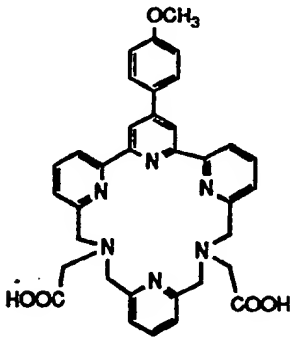

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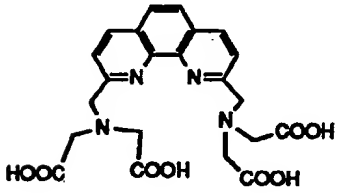
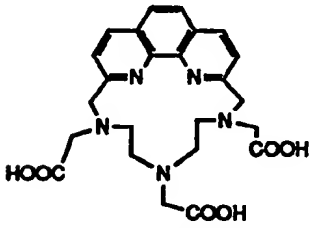


PheMT



MacroPheMT

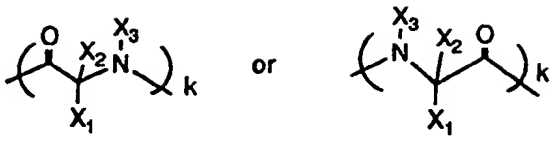
In one aspect, other suitable residues of chelating agents comprise proteins modified for the chelation of metals such as technetium and rhenium as described in U.S. Patent No. 5,078,985, the disclosure of which is hereby incorporated by reference.

In another aspect, suitable residues of chelating agents are derived from N_3S and N_2S_2 containing compounds, as for example, those disclosed in U.S. Patent Nos. 4,444,690; 4,670,545; 4,673,562; 4,897,255; 4,965,392; 4,980,147; 4,988,496; 5,021,556 and 5,075,099.

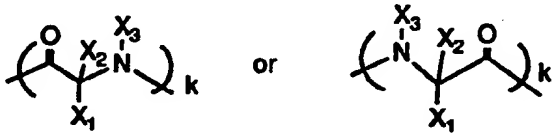
Other suitable residues of chelating agents are described in PCT/US91/08253, the disclosure of which is

hereby incorporated by reference. In structure V above, if W_1, W_2, and W_3 comprise the residue of multiple chelating agents, such agents can be linked together by a linking group such as described above in structure IV.

A residue of each of the chelating agents W_1, W_2, and W_3 in structure V is independently linked to the complementary L-enantiomeric oligonucleotide moiety cI or spacing group Q_{cI} through a chemical bond or a linking group, i.e., L_1, L_2 and L_3 in structure V, above. Preferred linking groups include nitrogen atoms in groups such as amino, imido, nitrilo and imino groups; alkylene, preferably containing from 1 to 18 carbon atoms such as methylene, ethylene, propylene, butylene and hexylene, such alkylene optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur or heteroatom-containing groups; carbonyl; sulfonyl; sulfinyl; ether; thioether; ester, i.e., carbonyloxy and oxycarbonyl; thioester, i.e., carbonylthio, thiocarbonyl, thiocarbonyloxy, and oxythiocarboxy; amide, i.e., iminocarbonyl and carbonylimino; thioamide, i.e., iminothiocarbonyl and thiocarbonylimino; thio; dithio; phosphate; phosphonate; urelene; thiourelene; urethane, i.e., iminocarbonyloxy, and oxycarbonylimino; thiourethane, i.e., iminothiocarbonyloxy and oxythiocarbonylimino; an amino acid linkage, i.e., a



group wherein k=1 and X_1, X_2, X_3 independently are H, alkyl, containing from 1 to 18, preferably 1 to 6 carbon atoms, such as methyl, ethyl and propyl, such alkyl optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur, substituted or unsubstituted aryl, containing from 6 to 18, preferably 6 to 10 carbon atoms such as phenyl, hydroxyiodophenyl, hydroxyphenyl, fluorophenyl and naphthyl, aralkyl, preferably containing from 7 to 12 carbon atoms, such as benzyl, heterocyclyl, preferably containing from 5 to 7 nuclear carbon and one or more heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; heterocyclylalkyl, the heterocyclyl and alkyl portions of which preferably are described above; or a peptide linkage, i.e., a



group wherein k>1 and each of X_1, X_2, and X_3 is independently represented by a group as described for X_1, X_2, and X_3 above. Two or more linking groups can be used, such as, for example, alkyleneimino and iminoalkylene. It is contemplated that other linking groups may be suitable for use herein, such as linking groups commonly used in protein heterobifunctional and homobifunctional conjugation and crosslinking chemistry as described for L_Z or L_Q in structure IV.

Especially preferred linking groups include unsubstituted or substituted phosphate ester groups containing amino groups which when linked to the residue of a chelating agent via an isothiocyanate group on the chelating agent form a thiourea group.

The linking groups can contain various substituents which do not interfere with the coupling reaction between chelate W_1, W_2, or W_3 and L-enantiomeric oligonucleotide cI or the spacing group. The linking groups can also contain substituents which can otherwise interfere with such reaction, but which during the coupling reaction, are prevented from so doing with suitable protecting groups commonly known in the art and which substituents are regenerated after the coupling reaction by suitable deprotection. The linking groups can also contain substituents that are introduced after the coupling reaction. For example, the linking group can be substituted with a group such as a halogen, such as F, Cl, Br or I; an ester group; an amide group; alkyl, preferably containing from 1 to about 18, more preferably, 1 to 4 carbon atoms such as methyl, ethyl, propyl, i-propyl, butyl, and the like; substituted or unsubstituted aryl, preferably containing from 6 to about 20, more preferably 6 to 10 carbon atoms such as phenyl, naphthyl, hydroxyphenyl, iodophenyl, hydroxyiodophenyl, fluorophenyl and methoxyphenyl; substituted or unsubstituted aralkyl, preferably containing from 7 to about 12 carbon atoms, such as benzyl and phenylethyl; alkoxy, the alkyl portion of which preferably contains from 1 to about 18 carbon atoms as described for alkyl above; alkoxyaralkyl, such as ethoxybenzyl; substituted or unsubstituted heterocyclyl, preferably containing from 5 to 7 nuclear carbon and heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; a carboxyl group; a carboxyalkyl

group, the alkyl portion of which preferably contains from 1 to 8 carbon atoms; or the residue of a chelating group.

In structure V, M_1, M_2 and M_3 each comprise elements with oxidation states equal to or greater than +1, and at least one of which is a radionuclide. Preferably each of M_1, M_2 and M_3 comprise a metal isotope, preferably a radioactive metal isotope, sometimes herein referred to as a metal radioisotope, which radioisotope is useful in a therapeutic or in a diagnostic imaging application. Preferred metal radioisotopes are selected from, for example, Sc, Fe, Pb, Ga, Y, Bi, Mn, Cu, Cr, Zn, Ge, Mo, Tc, Ru, In, Sn, Re, Sr, Sm, Lu, Eu, Ru, Dy, Sb, W, Re, Po, Ta and Tl. Useful emissions from such radioisotopes include spontaneous alpha emissions, beta emissions, gamma emissions, X-ray emissions, positron emissions, and such emissions as are induced by the processes of electron capture and internal conversion. Said emissions can be purely of one kind such as pure alpha, pure beta, pure gamma and the like, or of combinations of nuclear emissions such as beta and gamma emissions and the like.

Radioisotopes with emissions comprising, for example, alpha radiation or beta radiation are useful in therapeutic applications, especially in the therapy of cancer. Useful isotopes in therapeutic applications include, for example, alpha radiation emitting isotopes such as, for example, ^{207}Pb, ^{211}Pb, ^{208}Pb, ^{212}Pb, ^{212}Bi, ^{207}Ti, and ^{223}Ra; beta radiation emitting isotopes such as, for example ^{47}Sc, ^{66}Ga, ^{67}Cu, ^{77}As, ^{90}Y, ^{105}Rh, ^{109}Pd, ^{111}Ag, ^{121}Sn, ^{127}Te, ^{143}Pr, ^{149}Pm, ^{153}Sm, ^{161}Tb, ^{166}Ho, ^{169}Er, ^{177}Lu, ^{188}Re, ^{186}Re, ^{191}Os and ^{199}Au; and isotopes which emit radiation as a result of the processes of electron capture and internal conversion such as, for example, ^{97}Ru, ^{177m}Sn, ^{199}Sb, ^{128}Ba and ^{197}Hg. Radioisotopes especially preferred in

therapeutic applications include ^{212}Pb, ^{212}Bi, ^{90}Y, ^{177}Lu, ^{186}Re, and ^{188}Re. Currently the most preferred radioisotope is ^{90}Y.

Radioisotopes with emissions comprising, for example, gamma radiation or positron radiation are useful in diagnostic imaging applications, especially in diagnostic imaging of cancer. Useful isotopes in diagnostic imaging applications include, for example, gamma radiation emitting isotopes such as ^{47}Sc, ^{51}Cr, ^{67}Cu, ^{67}Ga, ^{97}Ru, ^{99m}Tc, ^{111}In, ^{117m}Sn, ^{141}Ce, ^{167}Tm, ^{199}Au, ^{87}Y and ^{203}Pb; and positron radiation emitting isotopes such as ^{44}Sc, ^{48}V, ^{64}Cu, ^{66}Ga, ^{69}Ge, ^{72}As, ^{86}Y and ^{89}Zr. Radioisotopes especially preferred in diagnostic imaging applications include ^{64}Cu, ^{99m}Tc, ^{111}In and ^{87}Y. Currently, the most preferred are ^{99m}Tc and ^{111}In.

In another aspect, other suitable radionuclides can be incorporated, for example, by covalent bonding into Q_{CI} and include radioactive isotopes of halogens such as radioactive isotopes of iodine, for example, ^{123}I, ^{124}I, ^{125}I and ^{131}I as well as radioactive isotopes of astatine such as ^{211}At.

Methods of generating an image useful in the diagnostic imaging of, for example, cancer in a mammal comprise detecting emissions imagewise from radioisotopes as employed in the compositions and methods of this invention. Said image generating methods comprise the use of, for example, a collimated camera detector such as a gamma camera commonly employed in radioimmunoscintigraphy (RIS), and the use of linked X-ray detectors commonly employed in positron emission tomography (PET) and in single photon emission tomography (SPET).

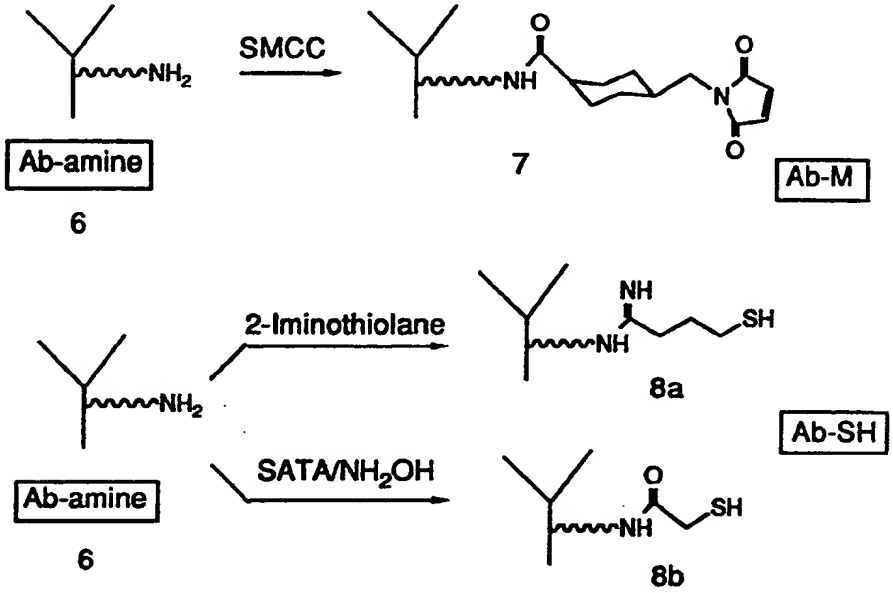
In structure V, x, y, and z are independently zero or 1 provided that at least one of x, y, or z is one; and

w and b are zero or an integer from 1 to about 4.

Preferred compositions can be prepared as outlined in the schemes that follow.

Scheme 6

Derivatization of antibody amine groups with heterobifunctional linking reagents SMCC, 2-IT, or SATA.



In scheme 6, the protein (antibody such as ING-1, antibody fragment, enzyme, receptor) is chemically modified for later covalent coupling to a thiolated L-enantiomeric oligonucleotide (to Ab-M) or to a maleimido-group-containing L-enantiomeric oligonucleotide (to Ab-SH). Chemical modification is effected using a bifunctional cross linking agent, preferably a heterobifunctional cross linking agent having both a group capable of reacting with protein functional groups (e.g. amine in Ab-amine) and also having a further group capable of reacting with thiol groups. The latter is selected from haloacetyl, halo-

acetamidyl, maleimido, and activated disulfide functions.

Maleimido and thioalkyl groups are introduced to an antibody by utilizing the heterobifunctional linkers, sulfosuccinimido-4-(M-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC), 2-iminothiolane (2-IT), or succinimidyl-S-acetylthioacetate(SATA). The reaction of a sample containing antibody with a linking agent is for a time sufficient to introduce an average of about 0.5-3 linking agent molecules per antibody molecule in the sample. The derivatized antibody is purified using a gel filtration column, and more preferably a Sephadex G-25 column.

Non-limiting examples of preferred protein conjugates are listed below:

ING-1-NH-CO-cyclohexane-CH_2-Maleimide;

ING-1-NH-C(=NH)-$(CH_2)_3$-SH; and

ING-1-NH-CO-CH_2-SH.

ING-1 is a preferred protein containing amine groups such as lysine amines as represented by Ab-amine.

Scheme 7

Derivatization of L-enantiomeric oligonucleotide "L-d(I-Q_I-I)" via amine groups with heterobifunctional linking reagents SATA and SMCC.

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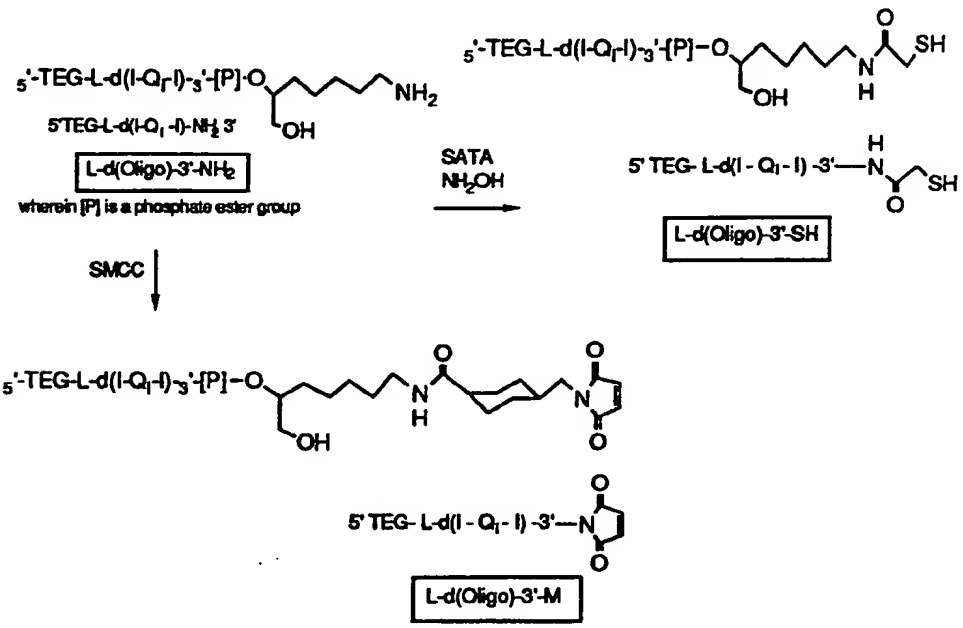
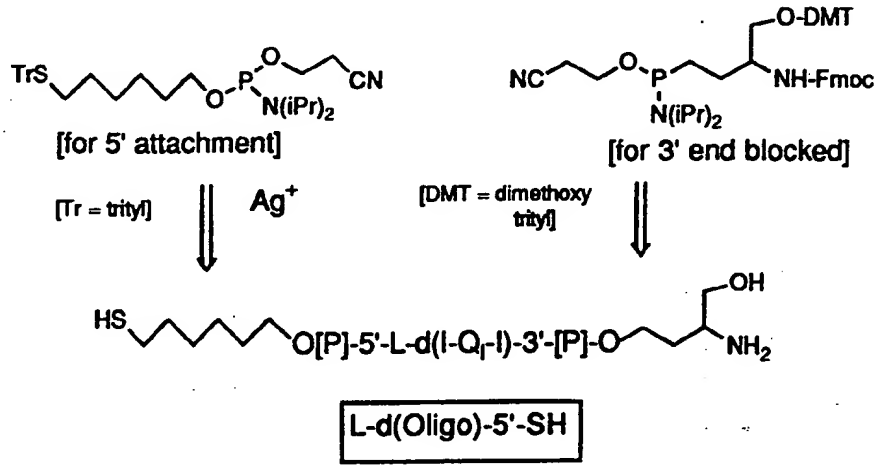


Scheme 8

Derivatization of L-enantiomeric oligonucleotide "L-d(I-Q_I-I)" with amine groups and with sulfhydryl groups for use with heterobifunctional linking reagents.

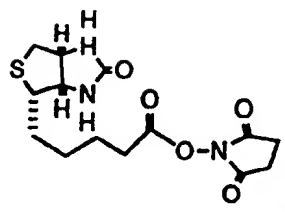


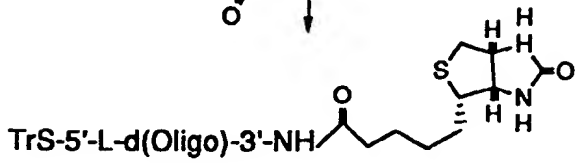
5'HS-L-d(I-Q_I-I)-NH_2 3'

Scheme 9

Derivatization of L-enantiomeric oligonucleotide "5'-TrS-L-d(I-Q_I-I)-3'-NH2" with biotin for use with heterobifunctional linking reagents.

TrS-5'-L-d(Oligo)-3'-NH_2



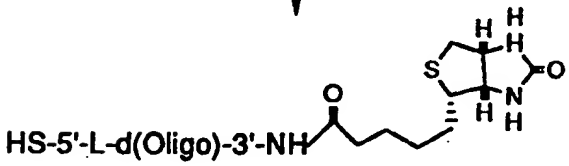


TrS-5'-L-d(Oligo)-3'-NH

TrS-5'-L-d(I-Q_I-I)-3'-Biotin

TrS-5'-L-d(Oligo)-3'-Biotin

Ag^+



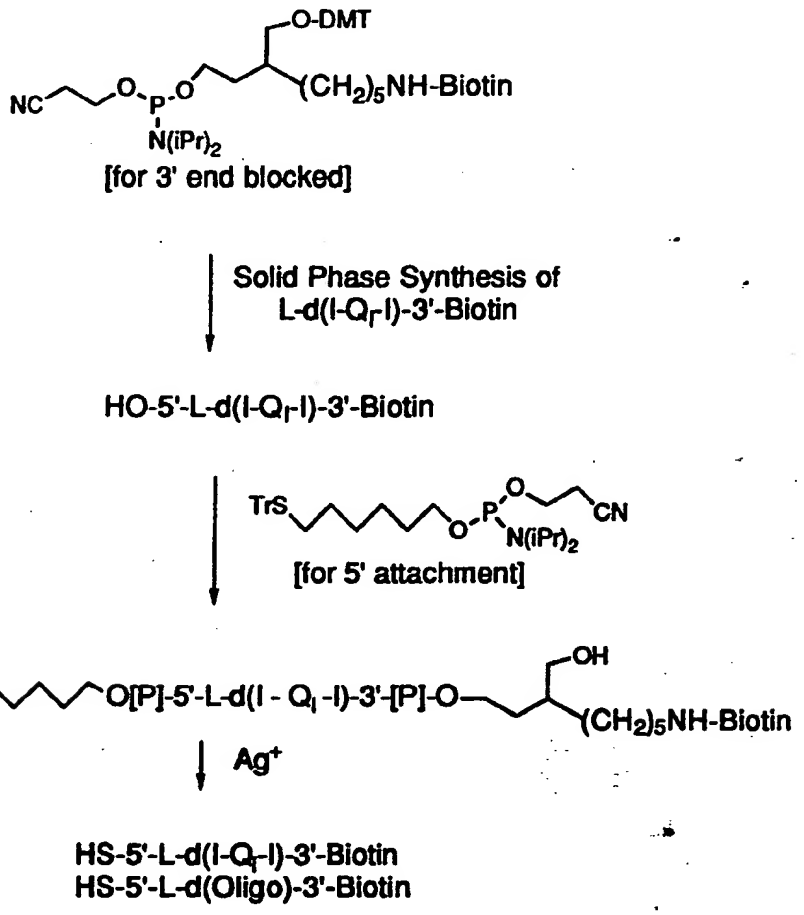
HS-5'-L-d(Oligo)-3'-NH

HS-5'-L-d(I-Q_I-I)-3'-Biotin

HS-5'-L-d(Oligo)-3'-Biotin

Scheme 10

Preparation of "5'-HS-L-d(I-Q_I-I)-3'-biotin" with biotinylated phosphoramidite reagent for use with heterobifunctional linking reagents.



L-enantiomeric oligonucleotides and modified L-enantiomeric oligonucleotides are synthesized according to standard methods such as solid phase synthesis that are well known in the art for the synthesis of D-enantiomeric oligonucleotides. Derivatizations of L-enantiomeric oligonucleotides L-d(I-Q_I-I) are achieved using the reaction of 5'-TEG-L-enantiomeric oligomer-NH_2-3' with SATA or SMCC to afford L-enantiomeric oligomer-3'-SH or L-enantiomeric oligomer-3'-M (see Scheme 7).

The following L-enantiomeric oligonucleotides are preferred:

(a) L-d(TTATGGACGGAGAAGCTAA) (SEQ ID NO: 8), and

(b) {5'-Teg-L-d(TCTTATGGACGGAGAAGCTAA-[ACTCTC]-TTATGGACGGAGAAGCTAATCT)-3'-amine-L-T}, (SEQ ID No:31)

wherein the square bracketed portion of SEQ ID No:31 denotes the preferred spacer, -L-d[ACTCTC]-, which separates two sequences each containing the preferred sequence -L-d(TTATGGACGGAGAAGCTAA)- (SEQ ID No: 8).

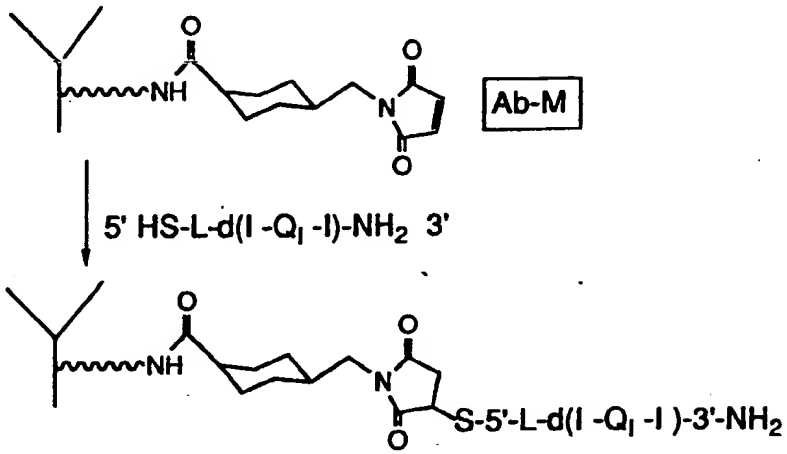
These L-enantiomeric oligomers are also derivatized to afford a bifunctionalized L-enantiomeric oligomer: 5'-HS-L-d(I-Q_I-I)-NH_2-3' via introduction of bifunctional reagents at the 5'- and 3'-positions, as shown in Scheme 8. The introduction of biotin at the 3'-position is also achieved by the reaction of 5'-HS-L-d(I-Q_I-I)-NH_2-3'with biotin-imidocarboxylate as shown in Scheme 9.

There are useful spacers with a protected amine, thiol, or carboxyl group on one end and a phosphoramidite at the other (see Scheme 10).

The applications of these spacers are shown in reactions below.

Scheme 11

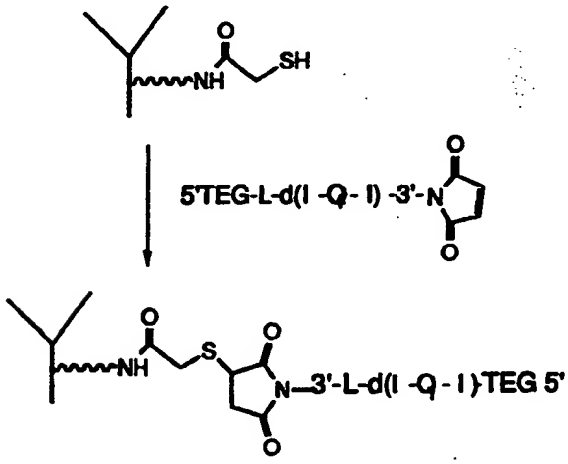
Assembly of Ab-M + L-d(Oligo)-5'-SH



Ab-M-5'-S-L-d(Oligo)-3'-NH$_2$

Scheme 12

Assembly of Ab-SH + L-d(Oligo)-3'-M



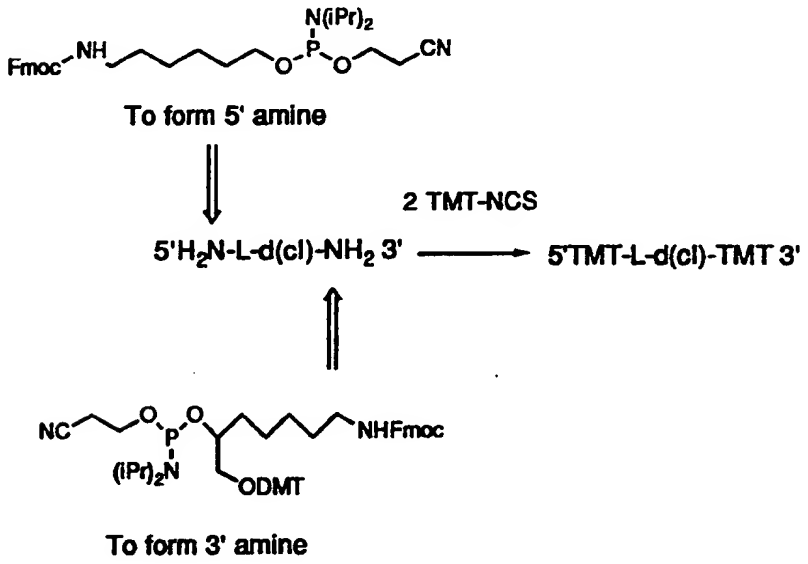
Ab-S-M-3'-L-d(Oligo)

The modified maleimido antibody (Ab-M, Scheme 6) and the thiolated L-enantiomeric oligonucleotide (5'-HS-L-d(I-Q$_I$-I)-NH$_2$-3') can be assembled to yield the modified antibody-L-enantiomeric oligonucleotide

conjugate (Ab-M-5'-S-succinimido-L-d(I-Q_I-I)-NH_2-3') as shown in Scheme 11. Similarly, Ab-S-M-3'-L-d(I-Q_I-I)-TEG-5' is prepared from Ab-SH and 5'-TEG-L-d(I-Q_I-I)-maleimide-3' as shown in Scheme 13.

Scheme 13

Complementary L-Oligonucleotide "5'-TMT-L-d(cI)-TMT-3' "



In these schemes, L-d(oligo) represents an L-enantiomeric oligodeoxyribonucleotide such as an L-deoxyribonucleotide, L-d(I); d(cI) represents the complementary L-enantiomeric oligodeoxyribonucleotide sequence; L-d(I-Q_I-I) is an L-enantiomeric oligodeoxyribonucleotide; and TMT represents a member of a class of terpyridine chelates, preferably as described above. A preferred TMT is 4'-(3-isothiocyanato-4-methoxyphenyl)-6,6"-bis[N,N-di(carboxymethyl)-aminomethyl]-2,2':6',2"-terpyridine, TMT-NCS.

The reaction of 5'-H_2N-L-d(cI)-NH_2-3' with 2 moles of TMT-NCS affords the desired 5'-TMT-L-d(cI)-TMT-3' as shown in Scheme 13.

There are other useful linking agents with a protected amine, thiol, or carboxyl group on one end and a phosphoramidite at the other. The applications of these agents are shown in the Examples.

In a preferred embodiment, an effective dose of a radioactive targeting reagent as described above in a pharmaceutically acceptable medium is prepared by exposing a composition comprising a complementary L-enantiomeric oligonucleotide sequence containing one or more chelating groups such as the oligodeoxyribonucleotide sequence as described above to a composition containing a radioactive metal isotope such that the molar amount of the radionuclide metal isotope is less than the molar amount of the chelating groups. The exposure lasts an effective time during which uptake of of the radionuclide metal isotope into the chelating agents occurs.

In a preferred embodiment, an effective dose of a non-radioactive targeting immunoreagent as described above in a pharmaceutically acceptable medium is administered to a patient and the non-radioactive targeting immunoreagent is allowed to accumulate at the target site such as at a tumor site in the patient. Subsequently, an effective dose of a radioactive targeting reagent as described above in a pharmaceutically acceptable medium is administered to the patient, and the radioactive targeting reagent is allowed to accumulate at the target site, said target site being the non-radioactive targeting immunoreagent which has accumulated at the tumor site in the patient.

The present invention also comprises one or more of the immunoreagents of this invention formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection for oral

administration in solid or liquid form, for rectal or topical administration, or the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenously, intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, intravesically, intra-articularly, locally (in the form of powders, ointments or drops), or as a buccal or nasal spray.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propylene glycol, polyethylene glycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such

solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols, and the like.

Solid dosage forms such as tablets, dragees, capsules, pills and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedding compositions which can be used are polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents. Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and, therefore, melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers or propellants as may be required. Ophthalmic formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

Actual dosage levels of active ingredient in the compositions of the present invention may be varied so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

The total daily therapeutic dose of the compounds of this invention administered to a host in a single or divided dose may be in amounts, for example, of from about 100 picomol to about 5 micromols per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

In another embodiment, the present invention is directed to a method of diagnosis comprising the administration of a diagnostic imaging effective amount of the compositions of the present invention to a mammal in need of such diagnosis. A method for diagnostic

imaging for use in medical procedures in accordance with this invention comprises administering to the body of a test subject in need of a diagnostic image an effective diagnostic image producing amount of the above-described compositions. In this method, an effective diagnostic image producing amount of a non-radioactive targeting immunoreagent as described above in a pharmaceutically acceptable medium is administered to a patient and said non-radioactive targeting immunoreagent is allowed to accumulate at the target site such as at a tumor site in said patient. Subsequently, a diagnostic imaging effective dose of a radioactive targeting reagent as described above in a pharmaceutically acceptable medium is administered to said patient, and said radioactive targeting reagent is allowed to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said patient. The image pattern can then be visualized.

The total diagnostic imaging effective dose of the compounds of this invention administered to a host in a single or divided dose may be in amounts, for example, of from about 1 picomol to about 0.5 micromols per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the effective diagnosting imaging dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

In addition to human patients, the test subjects can include mammalian species such as rabbits, dogs,

cats, monkeys, sheep, pigs, horses, bovine animals and the like.

After administration of the compositions of the present invention, the subject mammal is maintained for an effective time which is a time period sufficient for the administered compositions to be distributed throughout the subject and to enter the targeted tissues of the mammal. A sufficient time period for the non-radioactive targeting immunoreagent is generally from about 1 hour to about 2 weeks or more and, preferably from about 2 hours to about 1 week. A sufficient time period for the radioactive targeting reagent such as the preferred ^{90}Y is generally measured in terms of half-life of the radionuclide and as such is in the range of from about 1 to about 10 half-lives or more and, preferably from about 2 hours to about 6 half-lives.

The following examples further illustrate the invention and are not to be construed as limiting of the specification and claims in any way. Specific embodiments of the invention are illustrated in the following examples.

EXAMPLES

Example 1

L-Enantiomeric Oligonucleotide Design

A targeting immunoreagent of this invention as described in Structure IV comprising an antibody Z, linking groups L_Z and L_Q, an L-enantiomeric oligonucleotide sequence I, a spacing group Q_I, a second sequence I_i, and an end capping group E is designed as follows utilizing anticipated complementary binding properties between complementary pairs of mirror image D-enantiomeric oligonucleotides as a model for analogous physical properties imputed to the desired L-enantiomeric oligonucleotide.

A sample mirror image, naturally occurring D-enantiomeric oligodeoxyribonucleotide with the following sequence (herein referred to as SEQ ID NO: 32) is analyzed for conformity to the criteria described previously for Structure IV wherein groups L_Z, I, Q_I, I_i, and L_Q and E are as represented below as D-enantiomers:

L_Z I_Z Q_I I_i L_Q E

5'D-

[TC|TTATGGACGGATCCGCTAA|TC|TTATGGACGGATCCGCTAA|TCT]3'

(mirror image D-enantiomer SEQ ID NO: 32)

Analysis of this D-enantiomeric oligonucleotide sequence using "oligo" computer software (National Biosciences) designed for D-enantiomers reveals it to contain 5 regions of self-complementarity. All such regions are hairpin loops with differing degrees of overlap. The sum of the negative free energy changes (dG's) for these self-complementary regions is -28.3 kcal/mole which predicts a melting temperature of 86°C in 1.0 molar salt for this D-enantiomeric oligonucleotide. The most stable region of self-complementarity (dG = -15 kcal/mol) contains 8 base pairs most of which correspond to a BAM-1 restriction site palindrome. In the presence of these regions of self-complementarity a D-enantiomeric oligonucleotide sequence complementary to this sequence cannot hybridize successfully to its full length. By inference, a mirror image L-enantiomeric oligonucleotide of the same sequence and an L-enantiomeric oligonucleotide sequence complementary to the L-enantiomeric oligonucleotide sequence can also not hybridize successfully to its full length in the presence of mirror image regions of self-complementarity. Modifications to the D-enantiomeric oligonucleotide sequence (as well as respective modifications to the mirror image L-enantiomeric oligonucleotide) are then made in accordance with the

criteria outlined for structure IV to replace selected bases in regions of self-complementarity such that excessive base pairing is removed in iterative analysis. Additional D-enantiomeric and additional L-enantiomeric nucleotides are also inserted into the respective spacer sequences to ensure that, in helical conformations, the respective terminal groups of two respective complementary sequences, D-cI and L-cI, when hybridized to the two D-enantiomer sequences I and II and to the two L-enantiomer sequences, respectively are orthogonal to each other.

In this manner, a new D-enantiomeric oligonucleotide (SEQ ID NO: 33) with the following sequence is designed. The mirror image L-enantiomer (SEQ ID NO: 34) is synthesized and the L-enantiomer is conjugated to an immunoreactive molecule (i.e., to an antibody such as ING-1):

D-Enantiomer Oligonucleotide

Lz I Q_I I_i L_Q E

5'X-D[TC|TTATGGACGGAGAAGCTAA|ACTCTC|TTATGGACGGAGAAGCTAA|TCTYT]-3'

(SEQ ID NO: 33)

L-Enantiomer Oligonucleotide

L-d(Lz I Q_I I_i L_Q E)

5'X-L-d(TC|TTATGGACGGAGAAGCTAA|ACTCTC|TTATGGACGGAGAAGCTAA|TCTYT)-3'

(SEQ ID NO: 34)

This L-enantiomeric oligonucleotide (SEQ ID NO: 34) comprises two copies of the same L-enantiomeric

nucleotide sequence (i.e., I = I_i = L-d(TTATGGACGGAGAAGCTAA); SEQ ID NO: 8) linked through 6 L-enantiomeric nucleotide spacer [Q_I = L-d(ACTCTC)]. The groups L_Z and L_{QE} at the 5' or the 3' end comprise groups X and Y. X and Y can each independently be an amine-containing group (such as, for example, those available from Clontech Industries, each of which amine-containing groups being sometimes hereinafter cryptically referred to as "NH_2" or as "amine") or one of X and Y is selected from an amine-containing group and the other is selected from one or more TEG groups as described above. Additionally, one of X and Y can also be a terminal hydroxyl group (in which case the 3'-YT in the above sequence becomes a deoxyribosyl-OH group on removal from the solid phase support, and more preferably X is a 5'OH group available by removal of a DMT group with acid at the end of the synthetic sequence before deprotecting the amine groups with ammonium hydroxide), or one of X and Y can also be a terminal phosphate ester group (in which case the 3'-YT or the 5'-X becomes a $-OPO_3H_2$ group or an ionized salt such as a sodium salt thereof), as desired. Such phosphate terminal groups can be made by treatment of the 5'-OH terminal group with 2-cyanoethyl N,N-diisopropyl chlorophosphoramidite at the end of the synthesis. Subsequent reaction with ammonium hydroxide and oxidation provides the terminal phosphate group at X. A 3'-phosphate group can be introduced after synthesis of the oligomer and its removal from the solid support by treatment of the 3-hydroxyl group with phosphoric acid anhydide followed by hydrolysis in water.

Thus, in one embodiment, this L-enantiomeric oligonucleotide, cryptically referred to as 5'-Teg-L-d(I-QI-I)-3'-amine, is:

5'Teg-L-d(TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAATCT)-3'-

NH_2-T, i.e., (SEQ ID NO: 31), or 5'-Teg-L-d(I-Q_I-I)-3'amine.

In another embodiment, this L-enantiomeric oligonucleotide, cryptically referred to as 5'-amine-L-d(I-Q_I-I)-3'Teg, is:

5'H_2N-L-d(TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAATCT-3'-Teg-T) or 5'-amine-L-d(I-Q_I-I)-3'-Teg or (SEQ ID NO: 35)

In another embodiment, this L-enantiomeric oligonucleotide, cryptically referred to as 5'-amine-L-d(I-Q_I-I)-3'OH, is:

5'H_2N-L-d(TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAATCT-3'-OH) or 5'-amine-L-d(I-Q_I-I)-3'-OH of (SEQ ID NO: 36)

In another embodiment, this L-enantiomeric oligonucleotide, cryptically referred to as 5'-amine-L-d(I-Q_I-I)-3'OPO_3H_2, is:

5'H_2N-L-d(TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAATCT-3'-OPO_3H_2) or 5'-amine-L-d(I-Q_I-I)-3'-OPO_3H_2 or (SEQ ID NO: 37).

Example 2

(2a) Synthesis of L-d(I-Q_I-I); 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 (SEQ ID NO: 31)

This L-enantiomeric oligonucleotide is prepared on an Applied Biosystems oligonucleotide synthesizer originally designed by the manufacturer to be used for the synthesis of naturally occurring D-enantiomeric oligonucleotides. The method applicable to the trityl-on protocol for the synthesis of naturally occurring D-enantiomeric oligonucleotides is used as directed by the equipment manufacturer but is modified to instead use L-2-deoxynucleotide phosphoramidite reagent precursors 5'-dimethoxytrityl L-cytidine-3'-O-phosphoramidite, 5'-dimethoxytrityl L-adenosine-3'-O-phosphoramidite, 5'-

dimethoxytrityl L-guanosine-3'-O-phosphoramidite, and 5'-dimethoxytrityl L-thymidine-3'-O-phosphoramidite prepared as described above rather than the D-enantiomers. Clonetech's Uni-link Amino Modifier is used as the precursor to the 3'-amine group. The TEG group, a tetra(ethylene glycol) phosphate diester linked in this invention by a phosphate ester bond to 5'-dimethoxytrityl L-thymidine-3'-O-phosphoramidite [which is the L-enantiomer (mirror image) of the reagent disclosed in WO/92/02534 which refers to a tetra(ethylene glycol) phosphate diester linked by a phosphate ester bond to 5'-dimethoxytrityl D-thymidine-3'-O-phosphoramidite] is used at the 5'-end of the strand. Following synthesis of the entire L-enantiomeric oligonucleotide, the base protecting groups and solid support are removed with ammonium hydroxide and the resulting 5'-protecting group is removed with 3% trichloroacetic acid. The L-enantiomeric oligonucleotide is desalted and further purified by elution down an OPEC Cartridge (Clonetech) with deionized water. Electrophoresis on a 12% polyacrylamide gel is used to further purify the L-enantiomeric oligonucleotide. The L-DNA band is visualized by ultraviolet light shadowing. It is cut out, minced, and extracted with a buffer comprising 10mM Tris HCl and 1mM EDTA at pH 7.5 at 4°C for 24 hours. The gel pieces are then removed by centrifugation, and the L-DNA is purified through a spun column of Sephadex G-25. The concentration of L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(2b) Synthesis of 5'-H_2N-L-d(I-Q_I-I)-3'-Teg (SEQ ID NO: 35)

This L-enantiomeric oligonucleotide is prepared on an Applied Biosystems oligonucleotide synthesizer by the trityl-off protocol otherwise used for D-enantiomers as

directed by the equipment manufacturer but modified to use L-2-deoxynucleotide phosphoramidite reagent precursors (i.e., 5'-dimethoxytrityl L-cytidine-3'-O-phosphoramidite, 5'-dimethoxytrityl L-adenosine-3'-O-phosphoramidite, 5'-dimethoxytrityl L-guanosine-3'-O-phosphoramidite, and 5'-dimethoxytrityl L-thymidine-3'-O-phosphoramidite as described above). Clonetech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier) is used as the precursor of the 5'-amine group. The TEG group is used as the terminal group at the 3'-end. The base protecting group and solid support are removed with ammonium hydroxide and the L-enantiomeric oligonucleotide is further purified by polyacrylamide gel electrophoresis.

(2c) Synthesis of 5'-Trityl-S-L-d(I-Q_I-I)-3'-NH_2

The L-enantiomeric oligonucleotide sequence of Example 2a minus the Teg group is prepared on an Applied Biosystems oligonucleotide synthesizer by the trityl-on protocol as directed by the equipment manufacturer using L-2-deoxynucleotide phosphoramidite reagent precursors (5'-dimethoxytrityl L-cytidine-3'-O-phosphoramidite, 5'-dimethoxytrityl L-adenosine-3'-O-phosphoramidite, 5'-dimethoxytrityl L-guanosine-3'-O-phosphoramidite, and 5'-dimethoxytrityl L-thymidine-3'-O-phosphoramidite as described above). Clonetech's Uni-link Amino Modifier is used as the precursor to the 3'-amine group. Clonetech's C6-ThioModifier is used as the precursor to the 5'-thiol group (which is herein referred to cryptically as 5-S) added to the sequence in place of TEG in Example 2a. Following synthesis of the whole L-enantiomeric oligonucleotide, the base protecting groups and solid support are removed with ammonium hydroxide. The L-enantiomeric oligonucleotide is desalted and further purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The concentration of

L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(2d) Preparation of fluorescent 5'Teg-L-d(I-Q_I-I)-3'-NH-Cy5.18

A sample (30 nmoles) of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2, prepared according to Example 2a, is evaporated to dryness and redissolved in 500 mL 0.1 M bicarbonate buffer at pH 9 using a vortex mixer. This sample is then added to a vial containing the dried succinimidyl ester of the dye Cy5.18 (Biological Detection Systems; Pittsburg PA). After thorough mixing the reaction is allowed to proceed at room temperature for one hour with frequent mixing. The product, 5'-Teg-L-d(I-Q_I-I)-3'-NH-Cy5.18, is purified by elution from a Sepahadex G-25 column with distilled water.

Example 3

(3a) Synthesis of cI diamine, 5'-NH_2-L-d(cI)-3'-NH_2

An L-enantiomeric oligonucleotide, L-d(cI), containing the L-enantiomeric nucleotide sequence 5'X-L-d(TTAGCTTCTCCGTCCATAAYT)-3'(SEQ ID NO: 38) complementary to the L-enantiomeric oligonucleotide I is prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 2a. The 3'- (Y) and 5'- (X) amine-containing groups are incorporated as directed by the equipment manufacturer using Uni-link Amino Modifier (Clonetech) for the precursor to the 3'-amine group, and Clonetech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier: Catalog # 5202) as precursor to the 5'-amine group. After final deblocking and removal from the solid support, the protecting groups are removed with ammonium hydroxide, and the amine-functionalized L-enantiomeric oligonucleotide is purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The L-enantiomeric

oligonucleotide is further purified by polyacrylamide gel electrophoresis or reverse-phase HPLC. The concentration of L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(3b) Synthesis of I diamine, 5'-NH_2-L-d(I)-3'-NH_2

An L-enantiomeric oligonucleotide, I, containing the L-enantiomeric nucleotide sequence 5'X-L-d(TTATGGACGGAGAAGCTAAYT)-3'(SEQ ID NO: 39) is prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 3a. The 3'- (Y) and 5'- (X) amine-containing groups are incorporated as directed by the equipment manufacturer using Uni-link Amino Modifier (Clonetech) for the precursor to the 3'-amine group, and Clonetech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier: Catalog # 5202) as the precursor to the 5'-amine group. After final deblocking and cleavage from the solid support, the protecting groups are removed with ammonium hydroxide, and the amine-functionalized L-enantiomeric oligonucleotide is purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The L-enantiomeric oligonucleotide is further purified by polyacrylamide gel electrophoresis or reverse-phase HPLC. The concentration of L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(3c) Synthesis of L-d(cI)

An L-enantiomeric oligonucleotide, cI, containing the L-enantiomeric nucleotide sequence 5'-L-d(TTAGCTTCTCCGTCCATAA)-3' (SEQ ID NO: 23) complementary to (I) is prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 2a. After final deblocking and cleavage from the solid support, the protecting groups are removed with ammonium hydroxide. The L-enantiomeric oligonucleotide is

purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The L-enantiomeric oligonucleotide is further purified by polyacrylamide gel electrophoresis. The concentration of L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(3d) Synthesis of L-d(I)

An L-enantiomeric oligonucleotide, I, containing the L-enantiomeric nucleotide sequence 5'-L-d(TTATGGACGGAGAAGCTAA)-3' (SEQ ID NO: 8) is prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 3c. After final deblocking and cleavage from the solid support, the protecting groups are removed with ammonium hydroxide, and the L-enantiomeric oligonucleotide is purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The L-enantiomeric oligonucleotide is further purified by polyacrylamide gel electrophoresis. The concentration of L-enantiomeric oligonucleotide is estimated using absorbance at 260 nm.

(3e) Preparation of fluorescent cI diamine, 5'-NH_2-L-d(cI)-3'-NH_2, and fluorescent I diamine, L-d(I)

A sample (30 nmoles) of 5'-NH_2-L-d(cI)-3'-NH_2, prepared according to Example 3a, is evaporated to dryness and redissolved in 500 mL 0.1 M bicarbonate buffer at pH 9 by vortexing. This sample is then added to a vial containing the dried N-hydroxysuccinimidyl ester of the dye Cy5.18 (Biological Detection Systems; Pittsburg PA). After thorough vortexing the reaction is allowed to proceed at room temperature for one hour with frequent mixing. CY5.18 labeled 5'-NH_2-L-d(cI)-3'-NH_2 is purified by elution from a Sepahadex G-25 column with distilled water.

A sample of Cy5.18 labeled I diamine, 5'-NH_2-L-d(I)-3'-NH_2 of Example 3b is prepared in the same manner.

Example 4

(4a) Annealing of L-d(cI) to 5'-Teg-L-d(I-Q_I-I)-3'-NH_2

A sample of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 (2 mmoles), prepared according to Example 2a, is mixed with aliquots containing increasing amounts of L-d(cI) prepared by the method of Example 3c, [from 100:5 to 100:200 of L-d(I-QI-I):L-d(cI)], in 6SSC buffer at room temperature. The mixtures are loaded into a sample cuvette and analysed by UV light (260 nm) in a Cary 13 instrument while the cuvette temperature is ramped up from 30°C to 90°C and then back down to 30°C. The presence of binary complexes (L-d(cI):5'-Teg-L-d(I-Q_I-I)-3'-NH_2 and/or 5'-Teg-L-d(I-Q_I-I)-3'-NH_2:L-d(cI)) are indicated at concentrations of L-d(cI) below equimolar with respect to 5'-Teg-L-d(I-Q_I-I)-3'-NH_2. At higher concentrations of L-d(cI), a ternary complex (L-d(cI):5'-Teg-L-d(I-Q_I-I)-3'-NH_2:L-d(cI)) can be observed.

(4b) Hybridization of TMT-cI-TMT to L-d(I-QI-I)

Similar experiments using L-d(cI)-TMT (see Example 6a below) demonstrate the annealing of TMT-L-d(cI)-TMT to 5'-Teg-L-d(I-Q_I-I)-3'-NH_2. A ternary complex is formed.

Example 5

Preparation of ^{35}S-labeled TMT-NCS

A suspension of about 40 mmoles of TMT amine (PCT US91/08253) in 650 mL methanol is stirred at room temperature and deionized water is added dropwise (about 70 mL) until a clear pale yellow solution develops. The solution is cooled in an ice bath to 10°C and about 60 mmoles ^{35}S-thiophosgene is added dropwise over about 3

minutes. A precipate of TMT isothiocyanate forms and the solution is stirred continuously for a further 2.5 hours. The solution and precipitate are concentrated to near dryness on a rotovap under reduced pressure (~ 15mm Hg) at room temperature. The near-dry solid is diluted further with 750 mL of methanol and is stirred until the solids appear homogeneous. The solid TMT isothiocyanate (TMT-NCS) is then collected by filtration and is rinsed repeatedly with methanol. The product is dried overnight in a vacuum chamber.

Example 6

(6a) Preparation 5'-H_2N-L-d(cI)-3'-NH_2 conjugated to a chelating agent, TMT-NCS, to form 5'-TMT-L-d(cI)-3'-TMT and preparation 5'-H_2N-L-d(I)-3'-NH_2 conjugated to a chelating agent, TMT-NCS, to form 5'-TMT-L-d(I)-3'-TMT.

To 300 nmoles of one of the L-enantiomeric oligonucleotides, either 5'-H_2N-L-d(cI)-3'-NH_2 solution of Example 3a or 5'-H_2N-L-d(I)-3'-NH_2 solution of Example 3b, in 500 microL of 1.0 M carbonate/bicarbonate buffer at pH 9.0 is added 12 mg of TMT isothiocyanate (PCT US91/08253). The reaction mixture is vortex mixed and kept at 37°C for 2 hours and at room temperature for overnight. The resulting reaction mixture is quenched with ethanolamine (15 microM) and the product is purified by Sephadex G-25 column chromatography using deionized water as the eluting solvent.

The number of TMT's per molecule of L-enantiomeric oligonucleotide diamine is quantified by an asssay using the time resolved fluorescence of Europium metal chelated to the TMT.

(6b) General procedure for labelling of Ing-1-TMT, 5'-TMT-L-d(cI)-3'-TMT, and 5'-TMT-L-d(I)-3'-TMT conjugates with fluorescent metals.

Binding of lanthanides such as europium (3^+) to chelating agents that contain an aromatic moiety held close to the co-ordination sphere can lead to "sensitized" fluorescence wherein light is absorbed through the aromatic system and the energy is transferred to the metal. The metal then produces emissions characterized by a very large Stokes shift and fluorescence lifetimes of up to several seconds. The fluorescence at 615 nm is measured at a time-delay of 400 microseconds after an excitation pulse at 340 nm. This time delay is useful for high sensitivity measurements since short lived background fluorescence is eliminated and up to a 1,500 fold enhancement in sensitivity over normal Eu-fluorescence is achievable.

In this method, a known amount of Ing-1-TMT, 5'-TMT-L-d(cI)-3'-TMT, or 5'-TMT-L-d(I)-3'-TMT conjugate is titrated with increasing amounts of added $EuCl_3$ in an aqueous buffer. Thus, one microliter of a solution containing 1-30 picomoles of the conjugate is added, in duplicate, to wells in a Costar EIA/RIA 96-well plate containing a precalculated amount of Tris.HCl buffer (pH 7.4). The buffer volume is derived by subtracting from 99 the volume in microliters of aqueous $EuCl_3$ (typically 10^{-4} M to 10^{-6} M in Tris.HCl buffer). The total volume in each well is thereby fixed at 100 microliters. Aqueous $EuCl_3$ is then added to the buffered solution of the conjugate. The plate is then covered and shaken at low speed for one hour. The time resolved fluorescence is then measured using a Delfia 1232 time-resolved fluorometer (Wallac Inc.) and the data are analyzed. It is found that each conjugated TMT molecule chelates one Europium ion and that both 5'-TMT-L-d(cI)-3'-TMT and 5'-

TMT-L-d(I)-3'-TMT conjugates bind two Europium ions per molecule of conjugate.

(6c) Preparation of ^{35}S-labeled TMT-L-d(cI)-TMT from 5'-H_2N-L-d(cI)-3'-NH_2 and ^{35}S-labeled TMT-NCS.

^{35}S-labeled TMT-NCS (prepared as in Example 5) is substituted for the 12 mg of TMT isothiocyanate (PCT US91/08253) in the method of Example 6a and the reaction carried out as described above. The number of TMT's per molecule of 5'-H_2N-L-d(cI)-3'-NH_2 is quantified by counting the TMT-L-d(cI)-TMT product in a liquid scintillation counter optimized to detect ^{35}S. From a knowledge of the concentration of derivatized 5'-H_2N-L-d(cI)-3'-NH_2 and the specific activity of ^{35}S-thiophosgene, the number of TMT molecules per 5'-H_2N-L-d(cI)-3'-NH_2 diamine may be calculated.

Example 7

(7a) Preparation of a Yttrium (^{90}Y) radiolabeled-L-enantiomeric oligonucleotides, ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y and ^{90}Y-TMT-L-d(I)-TMT-^{90}Y

A solution of the L-enantiomeric oligonucleotide TMT conjugates, either (TMT-L-d(cI)-TMT) from Example 6a or TMT-L-d(I)-TMT from Example 6a, in deionized water at room temperature is treated with a solution of $^{90}YCl_3$ (>500 Ci/mg; from Amersham Corp.) in 0.5 M sodium acetate buffer at pH 6.0 to a specific activity of 0.1 Ci/pmole for one hour at room temperature. The labeling efficiency is determined by removing 1.0 microliter of the sample and spotting it on to a Gelman ITLC-SG strip. The strip is developed in a glass beaker containing 0.1 M sodium citrate at pH 6.0 for a few minutes until the solvent front reached three-quarters of the way to the top of the paper. The strip is then inserted into a System 200 Imaging Scanner (Bioscan)

which had been optimized for ^{90}Y and which is controlled by a Compaq 386/20e computer. In this system unbound ^{90}Y migrates at the solvent front. The TMT's of the L-enantiomeric oligonucleotide TMT conjugates chelate in excess of 98 % of the added radioactivity.

(7b) Hybridization of radiolabeled ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y or of ^{90}Y-TMT-L-d(I)-TMT-^{90}Y to 5'-Teg-L-d(I-Q_I-I)-3'-NH_2

A 5 pmole sample of ^{90}Y-TMT-L-d(cI)-TMT-90Y from Example 7(a) is mixed with increasing amounts (0.375 to 12 pmoles) of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 from Example 2a in PBS at 37°C for one hour. A 5 mL aliquot from each of these hybridizations is removed, mixed with SDS-containing buffer, and run on a 12% PAGE gel. Autoradiographs of the gels reveal that 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 is capable of binding to 2 molecules of cI.

Similar procedures using non-complementary, ^{90}Y-TMT-L-d(I)-TMT-^{90}Y from Example 7(a) fail to show hybridization to 5'-Teg-L-d(I-Q_I-I)-3'-NH_2.

(7c) Administration of ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y to Nude Mice

TMT-L-d(cI)-TMT labeled with ^{90}Y to a specific activity of 28 mCi/28 mg is injected into a 25 g nude mouse bearing a subcutaneous tumor in its right flank. At time intervals after injection, blood samples are taken from the tail and counted for ^{90}Y radioactivity in a liquid scintillation counter. The results reveal that more than 95% of the injected dose of ^{90}Y is removed from the blood stream in the first 30 minutes following injection. After 2 hours following injection the radioactivity in the blood levels off and a minute fraction (< 0.01% of injected dose) continues to circulate during the next 22 hours. These data demonstrate that in the absence of a hybridizing site

recognizing the L-d(cI) sequence, radiolabeled TMT-L-d(cI)-TMT is lost quickly from the circulation.

Example 8

(8a) Preparation of Antibody-Maleimide (Ab-M) using Sulfo-SMCC and ING-1; (ING-1-Maleimide).

A Sulfo-SMCC solution (108 nmoles) in phosphate buffered saline (PBS) is added to a sample of a chimeric antibody (ING-1; 18 nmoles) solution in phosphate buffer (pH 7). The resulting mixture is allowed to stand for 30 minutes with occasional mixing at room temperature. The reaction is stopped with 60 nmoles basic tris buffer. The reaction mixture is diluted with phosphate buffered saline, added to a prewashed PD-10 column, and eluted with PBS to afford ING-1-maleimide. This material is stored on ice until use.

(8b) Preparation of mercaptoalkyl-Antibody (Ab-SH) from ING-1 and 2-iminothiolane; (ING-1-SH)

A sample of a chimeric antibody (ING-1; 5 nmoles) solution in 0.1 M carbonate buffer (pH 8.8) is mixed with 200 nmoles of an aqueous solution of 2-iminothiolane. The resulting mixture is allowed to stand for 30 min with occasional mixing at room temperature. The reaction mixture is diluted with phosphate buffed saline, added to a prewashed PD-10 column (Pharmacia), and eluted with PBS to afford mercaptoalkyl-ING-1. This material is stored on ice until use.

Example 9

(9a) Preparation of a mercaptoalkyl-L-d(I-Q_I-I) using 2-iminothiolane; 5'-Teg-L-d(I-Q_I-I)-3'-SH

A sample of a solution of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 (30 nmoles) in water is mixed with 1 M carbonate buffer (pH 9) to give a final buffer concentration of 890 mM.

Into the buffered L-DNA is added 12 mmoles of an aqueous solution of 2-iminothiolane hydrochloride. These reactants are vortex mixed and kept at 37°C for 30 minutes. The reaction mixture is quenched by the addition of 12 mmoles of ethanolamine, diluted with phosphate buffed saline, added to a prewashed NAP-25 column (Pharmacia), and eluted with PBS to afford 5'Teg-L-d(I-Q_I-I)-3'-NH-C(N=H_2^+)$CH_2CH_2CH_2SH$ (as the hydrochloride Cl^-). For use in conjugation to a maleimide-derivatized antibody (Ab-M), the product is eluted from the column directly into the antibody solution. Otherwise, the mercaptoalkyl-L-d(I-Q_I-I) is stored on ice until use.

(9b) Preparation of a mercaptoalkyl-L-d(I-Q_I-I) using 2-iminothiolane; 5'-HS-L-d(I-Q_I-I)-3'-Teg

A sample of 5'-H_2N-L-d(I-Q_I-I)-3'-Teg (30 nmoles) is treated as in Example 9a to afford 5'-HS-$CH_2CH_2CH_2C(=NH_2^+)$-HN-L-d(I-Q_I-I)-Teg-3' (Cl^-).

(9c) Preparation of an L-d(I-Q_I-I)-Maleimide using Sulfo-SMCC; 5'-Teg-L-d(I-Q_I-I)-3'-Maleimide

An aqueous solution containing 20 nmoles of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 (prepared as in Example 2a) is diluted into phosphate buffed saline. Sulfo-SMCC (100 nmoles) in PBS is added and the resulting mixture is allowed to stand for 30 min with occasional mixing at room temperature. The reaction mixture is diluted with phosphate buffed saline, added to a prewashed PD-10 column, and eluted with PBS to afford 5'-Teg-L-d(I-Q_I-I)-3'-Maleimide. This material is stored on ice until use.

(9d) Preparation of an L-d(I-Q_I-I)-Maleimide; 5'-Maleimide-L-d(I-Q_I-I)-3'-Teg.

5'-H_2N-L-d(I-Q_I-I)-3'-Teg is reacted with sulfo-SMCC in the same manner as 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 in 9c to afford 5'-Maleimide-L-d(I-Q_I-I)-3'Teg.

(9e) Preparation of 3'-mercapto-L-d(I-Q_I-I) using SATA

An aqueous solution containing 50 nmoles of 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 (prepared as in Example 2a) is diluted into PBS and 500 nmoles of SATA (in DMSO) is added. After mixing and standing at room temperature for 60 min, the reaction mixture is diluted with PBS and eluted from a NAP-10 column with PBS to afford 5'-Teg-L-d(I-Q_I-I)-3'NH-CO-CH_2-S-CO-CH_3. The sulfhydryl group of the acetylthioacetylated L-enantiomeric oligonucleotide is deacylated by the addition of 30 mL of a pH 7.5 solution containing 100 mM sodium phosphate, 25 mM EDTA, and 500 mM NH_2OH. The reaction is allowed to proceed for two hours at room temperature after which time the material is passed down a NAP-5 column using PBS for the elution. The product, 5'-Teg-L-d(I-Q_I-I)-3'NH-CO-CH_2-SH, is used immediately to obviate oxidative dimerization.

(9f) Preparation of 5'mercapto-L-d(I-Q_I-I) using SATA

H_2N-5'-L-d(I-Q_I-I)-3'-Teg is reacted with SATA in the same manner as 5'-Teg-L-d(I-Q_I-I)-3'-NH_2 in Example 9e to afford 5'-HS-CH_2-OC-NH-L-d(I-Q_I-I)-3'-Teg.

Example 10

(10a) Conjugation of 5'Teg-L-d(I-Q_I-I)-3'-NH-C(=NH_2^+)$CH_2CH_2CH_2SH$ to Ab-M; ING-1-Maleimido-3'-S-L-d(I-Q_I-I)-5'-Teg

A sample (108 nmoles) of 5'-Teg-L-d(I-Q_I-I)-3'-NH-C(=NH_2^+)$CH_2CH_2CH_2SH$ (prepared according to Example 9a) is eluted with PBS from a NAP-25 column directly into a

solution of Ab-M (maleimide-derivatized ING-1; 18 nmoles) from Example 8a. After mixing, the reaction is allowed to proceed for 20 hours at 4°C. The reactants are then loaded into Centricon-100® ultrafiltration concentration devices (Amicon) which are then centrifuged at 1000 g for 25 minutes. The sample containing the product is resuspended in fresh PBS, and ultrafiltration, concentration by centrifugation and resuspension are repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm is constant. The final product is ING-1-Maleimido-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg.

(10b) Conjugation of 5'-$HSCH_2CH_2CH_2(NH_2^+=)C$-NH-L-d(I-Q_I-I)-Teg-3' to Antibody-Maleimide; ING-1-Maleimide-5'-S-L-d(I-Q_I-I)-Teg-3'

A sample (108 nmoles) of 5'-$HSCH_2CH_2CH_2(NH_2^+=)C$-NH-L-d(I-Q_I-I)-Teg-3' prepared according to Example 9b is conjugated to 18 nmoles of maleimide-derivatized ING-1 as in Example 8a to give ING-1-Maleimide-5'-S-$(CH_2)_3$-C(=NH_2^+)-NH-L-d(I-Q_I-I)-3'-Teg.

(10c) Assays on the ING-1-Maleimide-S-L-d(I-Q_I-I) conjugates

The optical density of each of the ING-1-Maleimide-S-$(CH_2)_3$-C(=NH)NH-L-d(I-Q_I-I) samples of example 10(a) and 10(b) is examined in a spectrophotometer at 260 nm and 280 nm. The ratio of optical densities at these two wavelengths is calculated, and, by using known extinction coefficients for the antibody and for the L-enantiomeric oligonucleotide (approximate molecular weight 16,500) at each of these wavelengths, the number of L-enantiomeric oligonucleotide molecules, L-d(I-Q_I-I), is estimated to be between 1 and 2 L-d(I-Q_I-I) per antibody in the sample.

The concentration of ING-1 in a conjugate solution is determined by the BioRad protein assay using bovine immunoglobulin as the protein standard. These data agreed well with the antibody concentrations determined by examination of the optical density of the conjugate at 280 nm once it has been corrected for absorbance due to the conjugated L-d(I-Q_I-I). Both these sets of data are further confirmed by subjecting the antibody-L-d(I-Q_I-I) conjugates to acid digestion and amino acid analysis.

Antibody-L-d(I-Q_I-I) conjugates are examined for their ability to bind to antigens on the surface of a human tumor cell line to which the antibody is raised. The immunoreactivity of the conjugates is compared by flow cytometry with a standard preparation of the antibody before being subjected to modification and conjugation to L-d(I-Q_I-I). Target HT29 cells (a human adenocarcinoma cell line: ATTC) are grown to confluency in tissue culture flasks using McCoy's media supplemented with 10% fetal calf serum. The cells are harvested by scraping the flask walls with a cell scraper. Cells from many separate flasks are pooled, centrifuged to a pellet, resuspended at 5 x 10^5/mL in a solution of ice-cold 50 mM sodium phosphate with 150 mM sodium chloride buffer pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide (Flow buffer). The cells are washed in this same buffer and then counted. An antibody standard curve is constructed by diluting a stock solution of ING-1 with an irrelevant (non binding), isotype-matched control antibody (human IgG_1) to give a number of samples ranging in ING-1 content from 10% to 100%. A standard curve is made in flow buffer so that each sample contains 1.0 mg antibody protein per mL. Samples from the standard curve and unknowns are then incubated with 5 x 10^5 HT29 cells at 4°C for 1 hour. After extensive

washing to remove unbound antibody, the cells are resuspended in 100 mL flow buffer and incubated at 4°C for 1 hour with goat-anti-human antibody labeled with fluorescene isothiocyanate (FITC). After further washing in flow buffer the samples are analyzed by flow cytometry on a Coulter EPICS 753 flow cytometer. Fluorescene from Fluorescene isothiocyanite (FITC) and propidium iodide (PI) is excited using the 488 nm emission line of an argon laser. The output is set at 500 mW in light regulation mode. Single cells are identified by 90 degree and forward angle light scatter. Analysis windows are applied to these parameters to separate single cells from aggregates and cell debris. Fluorescence from FITC and propidium are separated with a 550 nm long pass dichroic filter and collected through a 530 nm band pass filter (for FITC), and a 635 nm band pass filter (for PI). Light scatter parameters are collected as integrated pulses and fluorescence is collected as log integrated pulses. Dead cells are excluded from the assay by placing an analysis window on cells negative for PI uptake. The mean fluorescence per sample (weighted average from 2500 cells) is calculated for each histogram. FITC calibration beads are analysed in each experiment to establish a fluorescence standard curve. The average fluorescence intensity for each sample is then expressed as the average FITC equivalents per cell. Immunoreactivity is calculated by comparing the average fluorescence intensity of the unknown sample with values from the standard curve. From the immunoreactivity assay, ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'L-d(I-Q_I-I)-5'-Teg is approximately 2/3 as immunoreactive as the ING-1 standard and ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-5'-L-d(I-Q_I-I)-3'-Teg is approximately 4/5 as immunoreactive. In a separate set of experiments, immunoreactivity of ING-1-L-d(I-Q_I-I) (3'-conjugate) and ING-1-L-d(I-Q_I-I) (5'-conjugate) are

determined. Samples of these conjugates are also subjected to electrophoresis on Novex 6% polyacrylamide gels using SDS-containing buffers in order to estimate their apparent molecular weight and the degree of heterogeneity of the preparation. Using standards of known molecular weight run on the same gel, a standard curve is constructed of the distance travelled (Rf) versus the log of the molecular weight. From this standard curve the relative molecular weights of the bands associated with each conjugate preparation are determined. The SDS PAGE gels of ING-1-L-d(I-Q_I-I) and ING-1 antibody demonstrate that the molecular weight of the ING-1-L-d(I-Q_I-I) conjugates are higher than that of the antibody ING-1 alone.

(10d) Conjugation of Teg-5'-L-d(I-Q_I-I)-3'-Maleimide to mercaptoalkyl-Antibody, Ab-SH; ING-1-NH-CO-CH_2-S-Maleimido-3'-L-d(I-Q_I-I)-5'Teg

20 nmoles of Teg-5'-L-d(I-Q_I-I)-3'-Maleimide (from Example 9c) are reacted with 5 nmoles of mercaptoalkyl-ING-1 (Ab-SH from Example 8b) in PBS pH 7. After mixing, the reaction is allowed to continue at 4°C for 16 hours to afford ING-1-NH-CO-CH_2-S-Maleimido-3'-L-d(I-Q_I-I)-5'-Teg. The reaction mixture is loaded into a Centricon-100® ultrafiltration concentration device which is then centrifuged at 1,000 g for 25 minutes. The sample is resuspended in fresh PBS and this sequence of concentration by centrifugation is repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm is constant.

Maleimide-5'-L-d(I-Q_I-I)-3'-Teg is reacted with mercaptoalkyl-ING-1 (Ab-SH) in the same way to afford ING-1-NH-CO-CH_2-S-Maleimide-5'-L-d(I-Q_I-I)-3'Teg.

(10e) Conjugation of Teg-5'-L-d(I-Q_I-I)-3'-NH-C(=O)-CH_2-SH to Antibody-Maleimide, Ab-M.

A 6 nmole sample of ING-1-Maleimide (Ab-M from Example 8a) in PBS is reacted with 40 nmoles of Teg-5'-L-d(I-Q_I-I)-3'-NH-CO-CH_2-SH (from Example 9e) at 4°C for 16 hours. The reactants are diluted with PBS and eluted in PBS from a PD-10 column to afford ING-1-Maleimido-S-CH_2-C(=O)-NH-3'-L-d(I-Q_I-I)-5'Teg. The product is concentrated in a Centricon-300® device by centrifugation at 1000g for 25 minutes. The sample is resuspended in fresh PBS and concentration by centrifugation. This process is repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm is constant.

HS-CH_2-(O=)C-NH-5'-L-d(I-Q_I-I)-3'-Teg is reacted with ING-1-Maleimide in the same way to afford ING-1-Maleimido-S-CH_2-(O=)C-NH-5'-L-d(I-Q_I-I)-3'-Teg. The optical density of these samples is examined in a spectrophotometer at 260 nm and 280 nm. The ratio of optical densities at these two wavelengths is then calculated. By using known extinction coefficients for the antibody and for the L-enantiomeric oligonucleotide (approx molecular weight 16,500) at each of these wavelengths, the number of L-enantiomeric oligonucleotide molecules per antibody is estimated.

(10f) Conjugation of 5'-Trityl-(S)-L-d(I-Q_I-I)-3'-NH_2 to Antibody-Maleimide, Ab-M.

A sample (10 nmoles) of 5'-Trityl(S)-L-d(I-Q_I-I)-3'-NH_2 from Example 2c is diluted into PBS and a solution of silver nitrate in water is added to a final concentration of 85 mM. After vortexing, the reaction is allowed to proceed at room temperature for 30 minutes. A precipitate forms which is centifuged to the bottom of the tube. The clear supernatant which contains the L-enantiomeric oligonucleotide with a 5'

terminal thiol group and without the 5' trityl group (HS-5'-L-d(I-Q_I-I)-3'-NH_2) is kept at 4°C until use.

A 6 nmole sample of ING-1-Maleimide (from Example 8a) in PBS is reacted with 40 nmoles of HS-5'-L-d(I-Q_I-I)-3'-Teg at 4°C for 16 hours. The reactants are diluted with PBS and eluted in 4 mL from a pre-washed Econopac 106-DG column (BioRad) to afford ING-1-Maleimide-S-5'-L-d(I-Q_I-I)-3'-Teg. The product is concentrated in a Centricon-300® concentration device by centrifugation at 1000 g for 25 minutes. The sample is resuspended in fresh PBS and concentration by centrifugation is repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm is constant.

The optical density of these samples are examined in a spectrophotometer at 260 nm and 280 nm. The ratio of optical densities at these two wavelengths is calculated, and by using known extinction coefficients for the antibody and for the L-enantiomeric oligonucleotide (approximate molecular weight=16500) at each of these wavelengths, the number of L-enantiomeric oligonucleotide molecules per antibody is estimated.

Example 11

(11a) Annealing of L-d(cI) to ING-1-L-d(I-Q_I-I)

A sample of ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg (24 pmoles) from Example 10a and a sample of ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-5'L-d(I-Q_I-I)-3'-Teg (25 pmoles) from Example 10d are mixed in separate cuvettes with 16-fold excess of L-d(cI) from Example 3c in 50 mM PBS containing 1.0 mM EDTA and 100 mM NaCl, pH 7.2, at room temperature. The cuvettes are cooled to 20°C, loaded into a Cary 13 instrument, and the absorbance is analysed by UV light (260 nm) while the cuvette temperature is ramped up from 20°C to 80°C and then back down to 20°C at a rate of 0.5°C/min.

Analysis of the data reveals that cI is able to hydridize to both the 3'- and the 5'- conjugates to form a ternary complex with each. Similar experiments using TMT-L-d(cI)-TMT (from Example 6) demonstrate the annealing of TMT-L-d(cI)-TMT to ING-1-Maleimido-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg.

(11b) Hybridization of ING-1-L-d(I-Q_I-I) to either ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y or ^{90}Y-TMT-L-d(I)-TMT-^{90}Y in phosphate buffer and human serum

A solution of ING-1-Maleimido-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg (4 ul; 1 mg antibody/ml from Example 10a) and 50 microL ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y solution from Example 9a are mixed with freshly prepared human serum (200 mL) or PBS (200 mL; pH7.2) and incubated at 37°C for 2 hours. Aliquots are then subjected to SDS PAGE electrophoresis on an 8 to 16% gel. The gels are examined by both autoradiography and on a phosphoimager system to show that ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y is able to hybridize with ING-1-L-d(I-Q_I-I) in human serum as well as phosphate buffer solution (pH 7.2). Samples are left at room temperature for 14 days in PBS and in serum and run on 8 to 16% SDS-PAGE to give similar patterns to each other and to gels incubated at 37°C for 2 hours. This indicates that the conjugates are stable in serum for up to two weeks. ^{90}Y-TMT-L-d(I)-TMT-^{90}Y from Example 9a fails to show hybridization to ING-1-L-d(I-Q_I-I) at any time.

(11c) Hybridization of ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y to ING-1-L-d(I-Q_I-I)

A solution of ING-1-Maleimido-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg (4ul; 1mg/ml from Example 10a) and 50 uL ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y (from Example 9a) are mixed in PBS (200 ul; pH 7.2) and incubated at 37°C for 60 minutes. Aliquots of these mixtures are then

subjected to SDS PAGE electrophoresis on an 8 to 16% gel. The gels are autoradiographed on a phosphoimager system to show that ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y is able to hybridize with ING-1-L-d(I-Q_I-I).

(11d) Western blot to detect TMT on TMT-L-d(cI)-TMT hybridized with ING-1-L-d(I-Q_I-I).

A sample of L-d(I-Q_I-I) from Example 2a or of ING-1-Maleimido-3'-S-$(CH_2)_3$-C(=NH_2^+)-NH-L-d(I-Q_I-I)-5'-Teg from Example 10a is separately mixed with the L-enantiomeric oligonucleotide TMT conjugate TMT-L-d(cI)-TMT from Example 6. The reaction mixture of each is kept on ice for 10 minutes. Aliquots of each reaction mixture are mixed with SDS buffer and loaded onto two duplicate 8 to 16% polyacrylamide gels. The gels are subjected to electrophoresis at a constant voltage for 2 hours. One gel is electroblotted onto nitrocellulose paper using CAPS buffer for 20 minutes according to the manufacturer's protocol (Hoefer semi-dry transfer method). After washing thrice with a solution of 0.05% Tween 20 in PBS, the gel is blocked with a solution of 3% BSA in PBS at room temperature for 1 hour. Following further washing with Tween/PBS, the gel is overlaid with a solution of a murine anti-TMT antibody (10mg/ml in PBS/Tween) and left overnight at room temperature. The western blot is developed using a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (BioRad Western blot kit) and peroxidase substrate. The blot demonstrates that the TMT-L-d(cI)-TMT can be detected via the TMT's as being hybridized to bands that contained either L-d(I-Q_I-I) or ING-1-L-d(I-Q_I-I).

The second gel is stained with ethidium bromide (5mg/ml in distilled water). Examination of the stained gel under UV light again demonstrates hybrization.

Example 12

Preparation of Ab-TMT by direct conjugation (ING-1/TMT)

TMT-NCS (or a suitable derivative thereof) can be conjugated to an antibody molecule to yield an antibody-TMT conjugate molecule that displays the ability to bind to a target antigen recognized by the antibody variable region. Such a conjugate molecule can be used to deliver metal ions that are chelated by the TMT moiety in order to localize and/or treat the tumor that is targeted by such an immunoconjugate. In one preferred embodiment, the antibody is selected such that it has a broad reactivity with an antigen molecule expressed on tumor cells, thereby providing an antibody-TMT conjugate that can deliver radionuclides to the tumors for therapeutic or diagnostic purposes. The chimeric antibody, ING-1, (International patent publication WO 90/02569) consists of a murine variable region and a human immunoglobulin constant region. The antibody is produced by culturing a mouse myeloma cell line expressing the chimeric antibody essentially as described in the above-referenced publication. After purification, ING-1 is used at a concentration of 5.0 mg/mL in 50 mM sodium acetate and 150 mM sodium chloride buffered at pH 5.6.

The conjugation of ING-1 to TMT-NCS is achieved by first adding 1.0 M carbonate plus 150 mM sodium chloride buffer, pH 9.3, to ING-1 until the antibody solution reaches a pH of 9.0. A sample of that ING-1 solution containing 5 mg of protein is then pipetted into an acid washed, conical, glass reaction vial. A solution of TMT-NCS is prepared by dissolving 100 mg in 10 mL of 1.0 M carbonate plus 150 mM sodium chloride buffer, pH 9.0. The conjugation reaction is started by the addition of 96.5 mL of the TMT-NCS solution to the antibody to give a 4-fold molar excess of TMT-NCS over ING-1. The solution is stirred briefly to mix the

reactants and then left in the dark at room temperature. After 16 hours, the ING-1/TMT conjugate is separated from unconjugated TMT by applying the reaction mixture to a PD10 chromatography column which has been pre-washed and equilibriated with 50 mM sodium acetate in 150 mM sodium chloride buffer, pH 5.6. The pure conjugate is eluted from the column with 2.5 mL of that same buffer.

Example 13

Analytical tests on the ING-1/TMT conjugates.

(13a) Analysis of Chelator to Antibody Ratio

The protein concentrations of ING-1 in the conjugate solutions are determined by the BioRad protein assay using bovine immunoglobulin as the protein standard.

In order to calculate the number of functional TMT molecules per antibody, ING-1/TMT is reacted with a solution of Europium chloride until saturation of the metal-binding capacity of the TMT occurs. A 0.375 mg aliquot of the ING-1/TMT in 2.5 ml in 0.05 M Tris HCl buffer pH 7.5 is pipetted into a 5 ml quartz cuvette. A 20 mM Europium chloride (Europium chloride hexahydrate; Aldrich) solution in 0.05 M Tris HCl buffer pH 7.5 is prepared. An aliquot (50 mL) of this Europium chloride solution is added to the cuvette containing ING-1/TMT and the resulting solution is slowly stirred on a magnetic stirrer at room temperature for 10 min using a small magnetic stir bar placed in the cuvette. The fluorescence of the metal-ING-1/TMT complex is determined in a Perkin Elmer LS 50 spectrofluorometer using an excitation wavelength of 340 nm (10 nm slit width). The fluorescent emission is monitored at 618 nm using a 10 nm slit width and a 430 nm cutoff filter. The above procedure is repeated and fluorescent readings are made after each addition. Aliquots of Europium

chloride are added until the increase in fluorescence intensity is less than 5% of the preceding reading. A dilution correction is applied to the fluorescence intensity measured at each mole ratio to compensate for the change in volume of the test solution. Since each chelating site on the ING-1/TMT conjugate binds one Europium ion, and since Europium ion has to be in a chelate site for fluorescence to occur, this method allows the number of functional chelation sites to be quantitated.

Using this method, the ratio of TMT molecules per molecule of antibody is in the range from 0.3:1 to 2:1.

(13b) ING-1/TMT Immunoreactivity assay by ELISA

The antigen to which the antibody, ING-1, binds is prepared from LS174T or HT 29 cells (available from American Type Tissue Collection, ATTC) by scraping confluent monolayers of cells from the walls of culture flasks with a cell scraper. The cells from many flasks are combined and a sample is taken and counted to estimate the total number of cells harvested. At all times the cells are kept on ice. Following centrifugation of the cells at 1500 rpm for 10 minutes at 4°C, the cells are washed once in 25 mL ice-cold 50 mM sodium phosphate buffer supplemented with 150 mM sodium chloride, pH 7.4 (PBS), pelleted under the same conditions and transfered in 10 mL PBS to an ice-cold glass mortar. The cells are homogenized at 4°C using a motor-driven pestle and then centrifuged at 3000 x g for 5 minutes. The antigen-rich supernatant is removed from the other cell debris and subjected to further centrifugation at 100,000 x g for one hour at 4°C. The pellet (antigen fraction) from this final step is suspended in 100 mL of PBS for every million cells harvested. Following an estimate of the protein concentration (BioRad BCA protein assay using bovine

immunoglobulin as the protein standard) the antigen is stored at -20°C until use. Each well of a 96-well Costar microtiter plates is coated with antigen by adding 100 mL/well of cell lysate (10 mg/ml) prepared as above. The microtiter plates are allowed to dry overnight in a 37°C incubator. After washing the plates five times with 0.05% Tween-20 (Sigma) they are blotted dry. The wells of each plate are blocked by adding 125 mL/well of a 1% BSA (bovine serum albumin, Sigma A-7906) solution in PBS and incubated for 1 hour at room temperature. The plates are washed five times with 0.05% Tween-20. Samples (50 mL/well in duplicate) of ING-1/TMT conjugates and standard ING-1 antibody solutions are prepared at a range of concentrations in 1% BSA in PBS. Biotinylated ING-1 (1.0 mg/mL in 0.1% BSA) is added to each well (50mL/well) and the plates are then incubated for 2 hours at room temperature. Following five washes with 0.05% Tween-20, the plates are blotted dry and incubated at room temperature for one hour with dilute (1:2000 in 0.1% BSA) streptavidin-alkaline phosphatase (Tago; #6567). After a further five washes, color is developed in each well upon the addition of 100 mL per well of phosphatase substrate reagent (Sigma). After one hour at room temperature, the color is read using a 405 nm filter in a Titertek Multiscan microplate reader.

When tested by this procedure, the immunoconjugates of ING-1 with TMT are found to have immunoreactivity comparable to native ING-1.

(13c) ING-1/TMT Immunoreactivity Assay by Flow Cytometry

Target HT29 cells are grown to confluency in tissue culture flasks using McCoy's media supplemented with 10% fetal calf serum. The cells are harvested by scraping the flask walls with a cell scraper. Cells from many separate flasks are pooled, centrifuged to a pellet,

resuspended at 5 x 10^5/mL in a solution of ice-cold 50 mM sodium phosphate with 150 mM sodium chloride buffer pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide (Flow buffer). The cells are washed in this same buffer and then counted. An antibody standard curve is constructed by diluting ING-1 with an irrelevant (non binding), isotype-matched control antibody (human IgG1) to give a number of samples ranging in ING-1 content from 10% to 100%. The standard curve is made in flow buffer so that each sample contains 1.0 mg protein per mL. Samples from the standard curve and unknowns are then incubated with 5 x 10^5 HT29 cells at 4°C for 1 hour. After extensive washing to remove unbound antibody, the cells are resuspended in 100 mL flow buffer and incubated at 4°C for 1 hour with goat-anti-human antibody labeled with fluorescene isothiocyanate (FITC). After further washing in flow buffer the samples are analyzed by flow cytometry on a Coulter EPICS 753 flow cytometer. Fluorescene FITC and propidium iodide (PI) are excited using the 488 nm emission line of an argon laser. The output is set at 500 mw in light regulation mode. Single cells are identified by 90 degree and forward angle light scatter. Analysis windows are applied to these parameters to separate single cells from aggregates and cell debris. Fluorescence from FITC and propidium are separated with a 550 nm long pass dichroic filter and collected through a 530 nm band pass filter (for FITC), and a 635 nm band pass filter (for PI). Light scatter parameters are collected as integrated pulses and fluorescence is collected as log integrated pulses. Dead cells are excluded from the assay by placing an analysis window on cells negative for PI uptake. The mean fluorescence per sample (weighted average from 2500 cells) is calculated for each histogram. FITC calibration beads are analysed in each

experiment to establish a standard curve. The average fluorescence intensity for each sample is then expressed as the average FITC equivalents per cell. Immunoreactivity is calculated by comparing the average fluorescence intensity of the unknown sample with values from the standard curve. Samples of ING-1/TMT have immunoreactivity values comparable to the native ING-1 antibody by this method.

(13d) Determination of aggregate formation by size-exclusion HPLC.

A 30 cm x 7.5 mm TSK-G3000SW size-exclusion HPLC column (Supelco) fitted with a guard column of the same material is equilibrated with 12 column volumes of 10 mM sodium phosphate buffer pH 6.0 supplemented with 150 mM sodium chloride using a Waters 600E HPLC system with a flow rate of 1.0 mL per minute at 400-600 PSI. A sample (25 mL) of BioRad gel filtration protein standards is injected on to the column. The retention time of each standard is monitored by a Waters 490 UV detector set at 280 nm. Following the recovery of the final standard from the column, it is washed with a further 10 volumes of 10 mM sodium phosphate buffer, pH 6.0, supplemented with 150 mM sodium chloride. Samples (50mL) of either native ING-1 antibody or ING-1/TMT at 200 mg/mL are injected onto the column and their retention times are recorded. From the areas of the retained peaks and the retention time, the amount of aggregated material in the ING-1/TMT sample is calculated.

By this method the native ING-1 antibody has a retention time of 9.1 minutes. ING-1/TMT has a major peak also at 9.1 minutes but a minor peak, attributable to aggregates, can sometimes be seen at 7.3 minutes. By comparison of the peak areas, the aggregate peak is less than 5% of the total.

(13e) Radiolabeling of ING-1/TMT with ^{90}Y.

A volume of radioactive Yttrium chloride (^{90}Y in 0.04 M hydrochloric acid at a specific activity of >500 Ci/mg; Amersham-Mediphysics) is neutralized using two volumes of 0.5 M sodium acetate pH 6.0. The neutralized ^{90}Y (1.0 mCi) is added to 1.0 mL of ING-1/TMT (1 mg/mL) in 50 mM sodium acetate buffer containing 150 mM sodium chloride at pH 5.6. The labelling is allowed to proceed for one hour and then the reaction mixture is loaded onto a PD-10 chromatography column which has been pre-washed and equilibrated in a buffer containing 50 mM sodium phosphate with 150 mM sodium chloride pH 7.4 (PBS). The sample is eluted from the column with 1.5 mL of PBS. Fractions of radiolabeled ING-1/TMT (0.5 mL) are collected, assayed for radioactivity, and pooled. The labeling efficiency is determined by removing 1.0 mL of the sample and spotting it on to a Gelman ITLC-SG strip. The strip is developed in a glass beaker containing 0.1 M sodium citrate, pH 6.0, for a few minutes until the solvent front has reached three-quaters of the way to the top of the paper. The strip is inserted into a System 200 Imaging Scanner (Bioscan) which has been optimized for ^{90}Y and is controlled by a Compaq 386/20e computer. In this system free ^{90}Y migrates at the solvent front while the ING-1/TMT/^{90}Y remains at the origin.

Using this system more than 98% of the total ^{90}Y radioactivity is always found associated with ING-1/TMT at the origin.

(13f) Labeling ING-1/TMT with fluorescent metals.

Binding of lanthanides such as europium (3+) to chelating agents that contain an aromatic moiety held close to the co-ordination sphere can lead to "sensitized" fluorescence wherein light is absorbed through the aromatic system and the energy is transfered

to the metal. The metal then produces emissions characterized by a very large Stokes shift and fluorescence lifetimes of up to several seconds. A 0.5 mg aliquot of the ING-1/TMT in 2.5 mL in 0.05 M Tris HCl buffer pH 7.5 is pipetted into a 4 mL conical reaction vial containing a small stirring bar. A 250 mM europium chloride (europium chloride hexahydrate: Aldrich) solution in 0.05 M Tris HCl buffer pH 7.5 is prepared. An aliquot (50 mL) of this europium chloride solution is added to the reaction vial containing ING-1/TMT, and the resulting solution is stirred very slowly on a magnetic stirrer at room temperature. The labelling is allowed to proceed for one hour and then the reaction mixture is loaded on to a PD-10 chromatography column which had been pre-washed and equilibrated in a buffer containing 10 mM sodium phosphate and 150 mM sodium chloride at pH 6.0 (PBS). The sample is eluted from the column with 3.5 mL of PBS. The fluorescence of a 50 mL sample of the metal-ING-1/TMT complex is determined in a Perkin Elmer LS 50 spectrofluorometer using an excitation wavelength of 340 nm (10 nm slit width). The fluorescent emission is recorded at 618 nm using a 10 nm slit width and a 430 nm cutoff filter. Each functional chelating site on the ING-1/TMT conjugate binds one europium ion. Using this method, an average of between 0.1 and 3 fluorescent europium ions are bound per molecule of antibody in solution.

Example 14

(14a) Flow Cytometry of binding of fluorescent L-d(cI) to cells treated with ING-1-L-d(I-Q_I-I).

ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'Teg is prepared as in Example 10a. ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-5'-L-d(I-Q_I-I)-3'-Teg is prepared as in Example 10b. Fluorescently labeled CY5.18-L-d(I-Q_I-I) is prepared as in Example 2d. Fluorescently

labeled CY5.18-L-d(cI) and CY5.18-L-d(I) are prepared as in Example 3e. Flow cytometry is carried out essentially as described in Example 12c except that the fluorescent dye CY5.18 is used in place of FITC. HT-29 cells (0.5×10^6) are incubated on ice for 30 min with 1 mg each of the ING-1-L-d(I-Q_I-I) samples. The cells are washed twice with flow buffer and pelleted at 1400 rpm for 5 minutes between washes. Next the cells in each sample are incubated with 5 mg CY5.18-L-d(cI) or CY5.18-L-d(I) for 3 hours on ice. Some cells are incubated with CY5.18-L-d(cI) and CY5.18-L-d(I) alone. After extensive washing with flow buffer, the cells are subjected to analysis on a fluorescence activated cell sorter. CY5-18 calibration beads are analysed to establish a standard curve of relative fluorescence intensity versus CY5-18 concentration. The mean fluorescence per sample (weighted average from 2500 cells) is calculated for each histogram. The average fluorescence intensity for each sample is then expressed as the average CY5-18 equivalents per cell. Identical experiments are carried out in which the medium used for incubation of the cells with the components is 100% fetal calf serum in place of flow buffer.

The time taken to maximize hybridization of CY5-18-L-d(cI) to ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2+)-NH-3'-L-d(I-Q_I-I)-5'-Teg on the surface of HT-29 cells is about 3 hours. There is no difference between flow buffer and 100% FCS in the degree of hybridization in each preparation suggesting that the end-capped L-enantiomeric oligonucleotide strands are stable to nuclease digestion. Large amounts of CY5.18-L-d(cI) can hybridize to both conjugates bound to the surface of cells. There is relatively little non-specifc binding to the cells either by the fluorescent L-enantiomeric oligonucleotides themselves or by hybridization of CY5-18-L-d(I-Q_I-I) to the conjugates.

(14b) Binding of ^{90}Y-TMT-L-d(cI)-TMT-^{90}Y to HT-29 cells treated with ING-1-L-d(I-Q_I-I).

TMT-L-d(cI)-TMT is radiolabeled to a specific activity of 0.1 mCi/pmole as described in Example 7. ING-1-TMT-^{90}Y is prepared as in Example 13e. ING-1-Maleimide-S-$(CH_2)_3$-C(=NH_2^+)-NH-3'-L-d(I-Q_I-I)-5'-Teg is prepared as in Example 10a. Three tubes each containing 1 x 10^5 HT-29 cells in DMEM medium supplemented with 10% fetal calf serum are prepared and kept at 4°C throughout. Using matched protein concentrations in all tubes to ensure that the amount of added antibody is equal, the tubes are treated as shown in Table 1 below.

Table 1

Time (min)	Sample 1	Sample 2	Sample 3
0	+ING-1-L-d(I-Q_I-I)	0	0
60	Wash x2	Wash x2	Wash x2
90	+L-d(cI)-($TMT^{90}Y$)2	ING-1-$TMT^{90}Y$	Prehybridized ING-1-L-d-(I-Q_I-I) with L-d(cI)-($TMT^{90}Y$)2
150	Wash x2	Wash x2	Wash x2
180	Centrifuge cells and count pellet radioactivity	Centrifuge cells and count pellet radioactivity	Centrifuge cells and count pellet radioactivity

Both sample 1 and sample 3 show higher radioactivity associated with the cell pellet than does sample 2 (directly labeled ING-1-TMT-^{90}Y) suggesting that a delivery system consisting of 2 separate

(14c) Binding of Europium labeled ING-1/TMT and TMT-L-d(cI)-TMT conjugates to HT-29 cells and HT-29 cells treated with ING-1-L-d(I-Q_I-I), respectively

The TMT-L-d(cI)-TMT conjugate is labeled with Europium ions as described in Example 6b and ING-1/TMT is labeled with Europium ions as described in Example 13f. Standard curves are created for both conjugates in concentration ranges of 100 picomoles/100 microL to 6 femtomoles/100 microL. HT-29 cells are grown to confluence in McCoys media containing 10% FCS and 50 microgram/ml of gentamyacin. The cells are washed with phosphate buffered saline, and 5 ml of Trypsin Versene is added. The HT-29 cells are then incubated at 37°C in 5% CO_2 for 15 minutes, complete media (5 ml) is added, and the cells are removed and washed in PBS. The HT-29 cells are then blocked with 10 micrograms of sheared salmon sperm (natural D-enantiomer) DNA per 10^6 cells at 4°C for 30 minutes, washed in PBS and used in the hybridization assay as follows. 5 x 10^5 HT-29 cells are added to a 100 microL working dilution of Ing-1/TMT or Ing-1-L-d(I-Q_I-I) and the mixture is incubated for 30 minutes on ice. The cells are washed twice in 2 ml of a wash buffer (PBS + 0.1% BSA + 0.01% NaN_3) at 1400 RPM for 5 minutes. A working dilution of TMT-L-d(cI)-TMT (100 microL) is then added to the appropriate tubes and the reaction mixture is incubated on ice for 3 hours followed by washing twice in 2 mL of wash buffer (PBS + 0.1% BSA + 0.01% NaN_3) at 1400 RPM for 5 minutes.

Each binding study is done in triplicate. The cell suspensions after binding and hybridization as above are kept at 4°C in test tubes until use (10 minutes to one hour). The Europium fluorescence is measured in a Delfia 1232 time-resolved fluorimeter by aliquoting four 100 microL portions into separate wells in a Costar EIA/RIA 96-well plate from each tube after vortexing.

The results are processed as described in Example 15f. The counts per seconds (cps) of the Eu-TMT-L-d(cI)-TMT-Eu treated cells are considered as background and are subtracted from the cps data from the hybridization experiments (Eu-TMT-L-d(cI)-TMT-Eu/Ing-1-L-d(I-Q_I-I)). This result and the cps data from the Ing-1/TMT-Eu binding experiment are translated as picomoles of bound TMT molecules per cell from the individual standard curves created independently.

The results are described as fluorescence counts per second (cps) or number (#) of picomoles per 10^5 cells. ING-1/TMT-Eu and ING-conjugates: 0.25 microgram or 1.65 picomoles; Eu-TMT-L-d(cI)-TMT-Eu: 100 ng or 15 picomoles per 5 X 10^5 cells. For the same degree of modification of ING-1 by TMT and by L-d(I-Q_I-I) to form ING-1-TMT and ING-1-L-d(I-Q_I-I), respectively, an increase or amplification in fluorescence is seem in the latter when Eu is added to form ING-1/TMT-Eu from ING-1-TMT versus when Eu-TMT-L-d(cI)-TMT-Eu is added to ING-1-L-d(I-Q_I-I) to form the hybrid, respectively.

The invention has been described in detail with particular reference to certain preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Black, Christopher D.V.
Snow, Robert A.

(ii) TITLE OF INVENTION: TUMOR TARGETING WITH L-ENANTIOMERIC OLIGONUCLEOTIDE CONJUGATES OF IMMUNOREAGENTS AND OF CHELATED RADIONUCLIDES

(iii) NUMBER OF SEQUENCES: 39

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTATGGACGG AG 12

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..13
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTATGGACGG AGA 13

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTATGGACGG AGAA 14

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTATGGACGG AGAAG 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTATGGACGG AGAAGC 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..17
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTATGGACGG AGAAGCT 17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTATGGACGG AGAAGCTA 18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTATGGACGG AGAAGCTAA 19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGAGAAGCT AA 12

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..13

(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGGAGAAGC TAA 13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACGGAGAAG CTAA 14

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGACGGAGAA GCTAA 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGACGGAGA AGCTAA 16

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..17
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATGGACGGAG AAGCTAA 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TATGGACGGA GAAGCTAA 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTAGCTTCTC CG 12

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..13
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTAGCTTCTC CGT 13

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTAGCTTCTC CGTC 14

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTAGCTTCTC CGTCC 15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTAGCTTCTC CGTCCA 16

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..17
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTAGCTTCTC CGTCCAT 17

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTAGCTTCTC CGTCCATA 18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTAGCTTCTC CGTCCATAA 19

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCCGTCCAT AA 12

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..13
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTCCGTCCA TAA 13

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..14
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTCTCCGTCC ATAA 14

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..15

(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTTCTCCGTC CATAA 15

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCTTCTCCGT CCATAA 16

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..17
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGCTTCTCCG TCCATAA 17

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TAGCTTCTCC GTCCATAA 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals Teg."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2..50
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 51
(D) OTHER INFORMATION: /note= "N equals NH2."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 52
(D) OTHER INFORMATION: /note= "This nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT NT 52

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCTTATGGAC GGATCCGCTA ATCTTATGGA CGGATCCGCT AATCT 45

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 51
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT NT 52

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2..50
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 51
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 52

(D) OTHER INFORMATION: /note= "This nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT NT 52

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals H2N."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2..50
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 51
(D) OTHER INFORMATION: /note= "N Teg."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 52
(D) OTHER INFORMATION: /note= "This nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT NT 52

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals H2N."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2..50
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT 50

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals H2N."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2..50
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY:misc_feature
(B) LOCATION: 51
(D) OTHER INFORMATION: /note= "N equals OPO3H2."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

NTCTTATGGA CGGAGAAGCT AAACTCTCTT ATGGACGGAG AAGCTAATCT N 51

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 2..20
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 21
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 22
(D) OTHER INFORMATION: /note= "This nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

NTTAGCTTCT CCGTCCATAA NT 22

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 20
(D) OTHER INFORMATION: /note= "Each nucleotide is the L-enantiomeric form."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 21
(D) OTHER INFORMATION: /note= "N equals an amine-containing group."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 22
(D) OTHER INFORMATION: /note= "This nucleotide is the L-enantiomeric form."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

NTTATGGACG GAGAAGCTAA NT 22

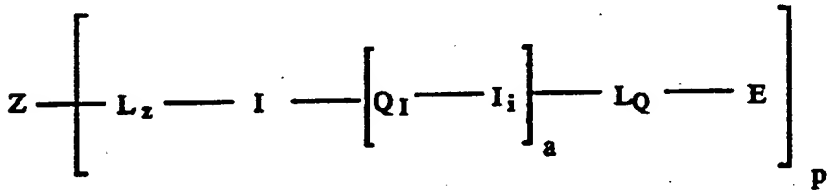
We claim:

1. A non-radioactive targeting immunoreagent that comprises a tumor antigen recognizing moiety, one or more non-self-associating L-enantiomeric oligonucleotide sequences, and one or more linking groups.

2. A radioactive targeting immunoreagent that comprises one or more chelating agents, one or more linking groups, one or more radionuclides, and an L-enantiomeric oligonucleotide sequence that is complementary in sequence to and capable of hybridization with one or more fragments of a non-self-associating L-enantiomeric oligonucleotide sequence.

3. A targeting immunoreagent that comprises moieties represented by the structure IV:

Structure IV



wherein:

Z is the residue of an immunoreactive protein;

L_Z and L_Q are independently a chemical bond or a linking group;

I is an L-enantiomeric oligonucleotide containing a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family containing from 12 to about 30 L-enantiomeric nucleotide units, provided that contiguous

sequences of six or more L-enantiomeric nucleotide units of said L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure IV;

Q_I is a spacing group;

a is 0 or an integer from 1 to about 6;

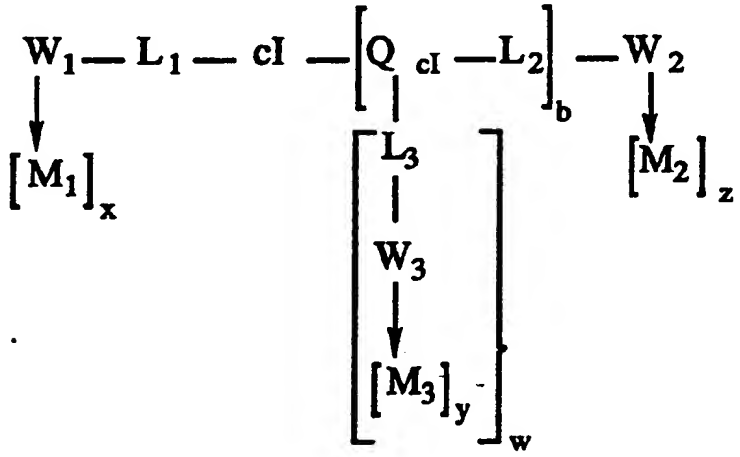
I_i is an L-enantiomeric oligonucleotide contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units, a contiguous sequence therein of which is identical to a contiguous sequence in I;

E is an end capping group; and

p is an integer from 1 to about 10.

4. A radioactive targeting reagent comprising moieties represented in the structure V:

Structure V



wherein:

cI is an L-enantiomeric oligonucleotide contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous

contiguous sequences, the individual homologs of which family comprise from 12 to about 30 L-enantiomeric nucleotide units; the L-enantiomeric nucleotide sequences of said homologs are complementary to L-enantiomeric nucleotide sequences of members of the set of L-enantiomeric oligonucleotides in a co-administerable targeting immunoreagent; and contiguous sequences of six or more L-enantiomeric nucleotide units of said complementary L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure V;

Q_{cI} is a spacing group;

L_1, L_2, and L_3 are each independently a chemical bond or a linking group;

W_1, W_2, and W_3 are each independently a residue of a chelating group;

M_1, M_2 and M_3 each comprise an element with an oxidation state equal to or greater than +1, and at least one of M_1, M_2 and M_3 is a radionuclide;

x, y, and z are independently zero or one provided that at least one of x, y, or z is one; and

w and b are independently zero or an integer from 1 to about 4.

5. The reagent of claim 3 wherein a is an integer from one to about 6.

6. The reagent of claim 3 wherein Z is the residue of an antibody or the residue of an antibody fragment.

7. The reagent of claim 6 wherein the antibody is selected from ING-1; B72.3; 9.2.27; D612; UJ13A; NRLU-10; 7E11C5; CC49; TNT; PR1A3; B174; C174; B43; and anti-HLB antibodies.

8. The reagent of claim 3 wherein L_Z or L_Q is a residue of a heterobifunctional cross-linking reagent.

9. The reagent of claim 8 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-iminothiolane, and N-succinimidyl S-acetylthioacetate.

10. The reagent of claim 3 wherein L_Z or L_Q is a residue of a modified nucleotide moiety comprising a reactive functional group.

11. The reagent of claim 10 where the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

12. The reagent of claim 3 wherein I and I_i are L-enantiomeric DNA.

13. The reagent of claim 3 wherein I has the sequence shown in SEQ ID NO: 8.

14. The reagent of claim 3 wherein Q_I is the residue of an L-enantiomeric oligonucleotide.

15. The reagent of claim 14 wherein the L-enantiomeric oligonucleotide has the sequence L-d(ACTCTC).

16. The reagent of claim 3 wherein E is the residue of a modified nucleotide group resistant to exonuclease activity.

17. The reagent of claim 16 wherein the modified nucleotide comprises a poly(alkylene glycol) phosphate diester.

18. The reagent of claim 17 wherein the diester is tetra(ethylene glycol) phosphate diester.

19. The reagent of claim 4 wherein cI has the sequence shown in SEQ ID NO: 23.

20. The reagent of claim 4 wherein Q_I is the residue of an L-enantiomeric oligonucleotide.

21. The reagent of claim 20 wherein the L-enantiomeric oligonucleotide has the sequence L-d(ACTCTC).

22. The reagent of claim 4 wherein L_1, L_2 or L_3 is the residue of a heterobifunctional cross-linking reagent.

23. The reagent of claim 22 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-iminothiolane, and N-succinimidyl S-acetylthioacetate.

24. The reagent of claim 4 wherein L_1, L_2 or L_3 is the residue of a modified nucleotide moiety containing a reactive functional group.

25. The reagent of claim 24 wherein the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

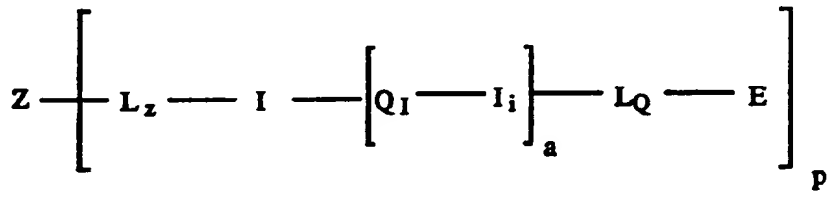
26. The reagent of claim 4 wherein W_1, W_2 or W_3 independently contains a polycarboxylic acid group.

27. The reagent of claim 4 wherein W_1, W_2 or W_3 is independently selected from the group consisting of a residue of B4A, P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT.

28. The reagent of claim 4 wherein the radionuclide is selected from ^{44}Sc, ^{64}Cu, ^{67}Cu, ^{111}In, ^{212}Pb, ^{68}Ga, ^{87}Y, ^{90}Y, ^{153}Sm, ^{212}Bi, ^{99m}Tc, ^{177}Lu ^{186}Re and ^{188}Re.

29. A method of making a compound of the structure:

Structure IV



wherein:

Z is the residue of an immunoreactive protein;

L_Z and L_Q are independently a chemical bond or a linking group;

I is an L-enantiomeric oligonucleotide containing a contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family containing from 12 to about 30 L-enantiomeric nucleotide units, provided that contiguous sequences of six or more L-enantiomeric nucleotide units

of said L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure IV;

Q_I is a spacing group;

a is an integer from 1 to about 6;

I_i is an L-enantiomeric oligonucleotide contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units, a contiguous sequence therein comprising a portion of I;

E is an end capping group; and

p is an integer from 1 to about 10;

comprising:

(i) derivatizing Z with a precursor to a residue of L_Z under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $Z-[L_Z]p$;

(ii) derivatizing a precursor to a residue of E with a precursor to a residue of L_Q under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_Q-E;

(iii) derivatizing a precursor to a residue of L_Q-E with a precursor to a residue of I_i under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of I_i-L_Q-E;

(iv) derivatizing a precursor to a residue of I_i-L_Q-E with a precursor to a residue of Q_I under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $Q_I-I_i-L_Q-E$;

(v) derivatizing a precursor to a residue of $Q_I-I_i-L_Q-E$ with a precursor to a residue of I_i and then with a precursor to a residue of Q_I under conditions and for a time period sufficient to form a covalent complex which is a precursor of a residue of $[Q_I-I_i]a-L_Q-E$;

(vi) derivatizing a precursor to a residue of $[Q_I-I_i]a-L_Q-E$ with a precursor to a residue of I under conditions

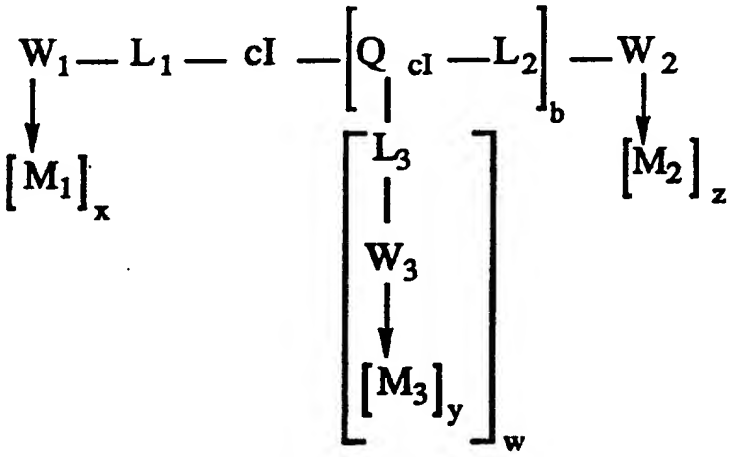
and for a time period sufficient to form a covalent complex which is a precursor to a residue of $I-[Q_I-I_i]_a-L_Q-E$;

(vii) derivatizing a precursor to a residue of $I-[Q_I-I_i]_a-L_Q-E$ with a precursor to a residue of L_z under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $L_z-I-[Q_I-I_i]_a-L_Q-E$; and

(viii) derivatizing a precursor to a residue of $L_z-I-[Q_I-I_i]_a-L_Q-E$ with a precursor to a residue of $Z-[L_z]_p$ under conditions and for a time period sufficient to form a covalent complex $Z-[L_z-I-[Q_I-I_i]_a-L_Q-E]_p$.

30. A method of making a compound of the structure:

Structure V



wherein:

cI is an L-enantiomeric oligonucleotide contiguous sequence of from 12 to about 50 L-enantiomeric nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of which family comprise from 12 to about 30 L-enantiomeric

nucleotide units; the L-enantiomeric nucleotide sequences of said homologs are complementary to L-enantiomeric nucleotide sequences of members of the set of L-enantiomeric oligonucleotides in a co-administerable targeting immunoreagent; and contiguous sequences of six or more L-enantiomeric nucleotide units of said complementary L-enantiomeric oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous L-enantiomeric nucleotide units anywhere in structure V;

Q_{cI} is a spacing group;

L_1, L_2, and L_3 are each independently a chemical bond or a linking group;

W_1, W_2, and W_3 are each independently a residue of a chelating group;

M_1, M_2 and M_3 each comprise an element with an oxidation state equal to or greater than +1, and at least one of M_1, M_2 and M_3 is a radionuclide;

x, y, and z are independently zero or one provided that at least one of x, y, or z is one; and

w and b are zero or an integer from 1 to about 4;

comprising:

(i) derivatizing a precursor to a residue of L_1 with a precursor to a residue of cI under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_1-cI;

(ii) derivatizing a precursor to a residue of Q_{cI} with a precursor to a residue of L_3 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of Q_{cI}-$[L_3]_w$;

(iii) derivatizing a precursor to a residue of L_1-cI with a precursor to a residue of Q_{cI}-$[L_3]_w$ under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_1-cI-$\{Q_{cI}$-$[L_3]_w\}$;

(iv) derivatizing a precursor to a residue of L_1-cI-{Q_{cI}-[L_3]$_w$} with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_1-cI-{Q_{cI}-[L_3]$_w$-L_2};

(v) derivatizing a precursor to a residue of L_1-cI-{Q_{cI}-[L_3]$_w$-L_2} with a precursor to a residue of Q_{cI}-[L_3]$_w$ and then with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of

$$L_1\text{-cI-}[Q_{cI} - L_2]_b;$$
$$|$$
$$[L_3]_w$$

(vi) derivatizing a precursor to a residue of

$$L_1\text{-cI-}[Q_{cI} - L_2]_b$$
$$|$$
$$[L_3]_w$$

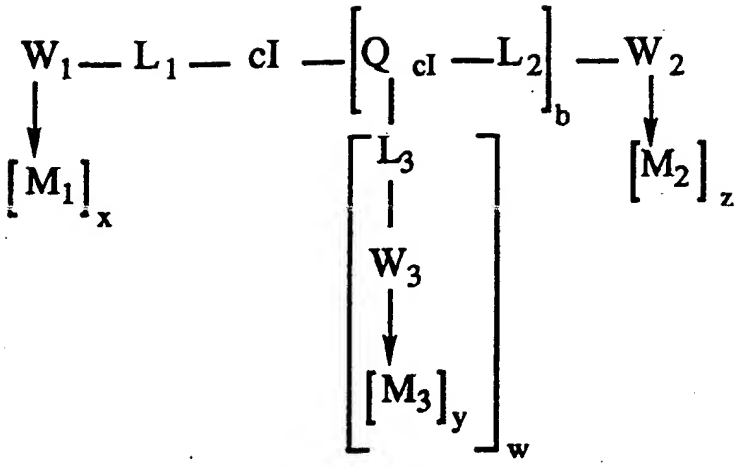
with a precursor to a residue of W_1, W_2, and W_3 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of

$$W_1\text{-}L_1\text{-cI-}[Q_{cI} - L_2]_b\text{-}W_2;$$
$$|$$
$$[L_3 - W_3]_w$$

(vii) derivatizing a precursor to a residue of

$$W_1\text{-}L_1\text{-cI-}[Q_{cI} - L_2]_b\text{-}W_2$$
$$|$$
$$[L_3 - W_3]_w$$

with a precursor to a residue of $[M_1]_x$, $[M_2]_z$, and $[M_3]_y$ under conditions and for a time period sufficient to form the compound



31. A pharmaceutical composition comprising a compound of claim 3 dissolved or dispersed in a pharmaceutically acceptable medium.

32. A pharmaceutical composition comprising a compound of claim 4 dissolved or dispersed in a pharmaceutically acceptable medium.

33. A method of treating a tumor in a mammal comprising administering to said mammal an effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the tumor site in said mammal, and subsequently, administering an effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said mammal, waiting for a time period sufficient for said radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said mammal.

34. A method of diagnostic imaging in a mammal comprising administering to said mammal an imaging effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the imaging site in said mammal, and subsequently, administering an imaging effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said mammal, waiting for a time period sufficient for said radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said imaging site in said mammal and generating an image of said mammal.

35. The reagent of claim 3 wherein I is a double helix.

36. The reagent of claim 4 wherein cI is a double helix.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02610

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C07K 3/08, 15/28; A61K 39/395, 43/00
US CL :530/391.1, 402; 424/1.11, 85.91
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/391.1, 402; 424/1.11, 85.91

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,863,713 (GOODWIN ET AL) 05 September 1989, col. 3, lines 30-68; col. 7, line 40; col. 9, lines 37-43.	1-33, 35-36
Y	US, A, 5,158,880 (EVELEIGH) 27 October 1992, col. 1, lines 23-28.	1-33, 35-36
Y	WO, A, 92/08494 (TONER ET AL) 29 May 1992, see claims 9 and 11 in particular.	1-33, 35-36
Y	Journal of the American Chemical Society, Volume 113, No. 21, issued 09 October 1991, H. Urata et al, "Mirror-Image DNA", pages 8174-8175, see entire document.	1-33, 35-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
16 MAY 1994	01 JUN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTINA CHAN Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02610

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-33 and 35-36

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02610

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-33 and 35-36, drawn to a non-radioactive targeting immunoreagent , a radioactive targeting immunoreagent, a process of making a non-radioactive targeting compound, a process of making a radioactive targeting compound, a composition and a method of treating a tumor in a mammal, classified in class 530, subclasses 391.1 and 402 and class 424, subclasses 1.11 and 85.91.

II. Claim 34, drawn to a method of diagnostic imaging in a mammal, classified in class 424 subclass 9.

The claimed inventions as grouped are distinct, each from the other, because they represent different inventive endeavors. The invention of Group I would not suggest the invention of Group II. Groups I and II are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.